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13. ABSTRACT *(Maximum 200 Words)*

The goal of this project is to understand adhesive mechanisms in breast cancer metastasis because this is the primary cause of death. Breast cancer metastasis to bone, lungs, liver and brain involves the blood stream and requires tumor cell arrest within the vasculature. In leukocytes and platelets, arrest within the vasculature depends on integrin activation. We found that breast cancer cells can express integrin αvβ3 in an activated or a non-activated state. Only activated αvβ3 supported breast cancer cell arrest during blood flow. The metastatic activity of breast cancer cells was strongly increased when αvβ3 was activated. To modulate αvβ3 function, we expressed β3 mutants in MDA-MB 435 breast cancer cells. $\beta3_{D723R}$ resulted in constitutively activated αvβ3 and a highly metastatic phenotype. To test the significance of integrin αvβ3 activation in human breast cancer, we isolated primary metastatic cells from breast cancer patients and found that all expressed αvβ3 in an activated state. These results validate our original hypothesis that the metastatic phenotype in human breast cancer involves activation of tumor cell adhesion receptor, integrin αvβ3. Consequently, alterations within tumors that lead to the aberrant control of αvβ3 activation are expected to adversely affect the course of human breast cancer.

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INTRODUCTION:

Complications from *metastatic disease* are the primary cause of death in breast cancer. The *purpose* of this project is to understand adhesive mechanisms in breast cancer metastasis because these may critically determine breast cancer progression and represent a target to combat the disease. We found that human breast cancer cells can express the adhesion receptor, integrin αvβ3 in an activated or a non-activated state. We *hypothesized that breast cancer cell integrin αvβ3 activation determines the metastatic phenotype of the tumor cells. Our results from the initial funding period validate this hypothesis.* The results indicate a causal relationship between breast cancer cell integrin αvβ3activation and an increase in metastatic activity. They provide information on a potential mechanism of αvβ3 activation and show a significance of integrin αvβ3 activation in primary metastatic cells from breast cancer patients.

BODY:

Integrin αvβ3 has been implicated in the pathophysiology of malignant tumors [1]. It clearly has a role on endothelial cells where it is required for tumor angiogenesis [2]. In several malignancies, however, αvβ3 was also found on the tumor cells, and expression correlates with tumor progression in melanoma, glioma, ovarian and breast cancer [3-7]. In breast cancer, αvβ3 expression characterizes the metastatic phenotype as this integrin is clearly upregulated in invasive tumors, particularly in bone metastases [8]. A mechanistic role of αvβ3 in the spread of breast cancer, however, has yet to be established. We previously suggested that an interaction of circulating tumor cells with platelets represents a potential mechanism for tumor cell arrest within the vasculature [9]. To test the hypothesis that tumor cell binding to platelets during blood flow is a critical ability of metastatic cells, we injected MDA-MB 435 human breast carcinoma cells into the mammary fat pads (mfp) of SCID mice and retrieved cells from the resulting tumors and from metastases to lymphnodes, lungs, bone and the pleural cavity. We compared these variant cells by blood perfusion studies *in vitro* for their ability to attach to activated platelets and undergo platelet mediated arrest. MDA-MB 435 parental cells largely failed to adhere or interact with platelets under dynamic blood flow conditions. In contrast, cell variants derived from mfp tumors and distant metastases readily adhered and used platelet interaction for cell arrest (Fig. 1A). Importantly, metastatic cells freshly isolated from a pleural effusion of a patient with advanced breast cancer (PE02JA), also exhibited a very strong platelet interactive phenotype as these cells attached to platelets and were incorporated into thrombi formed at a collagen type I matrix during blood perfusion (Fig. 1A). Using the clinical sample, Fig. 1B illustrates the typical morphology of breast cancer cell binding to activated platelets, as observed in the blood perfusion studies. The interaction of the breast cancer cells with platelets depended on the function of tumor cell integrin αvβ3 and platelet integrin αIIbβ3 (Fig. 2). Tumor cell binding was inhibited by function blocking antibodies directed against integrin αvβ3 (Fig. 2A) or platelet receptor αIIbβ3 (Fig. 2B). Similar results were obtained for all variants of MDA-MB 435 cells derived from mfp tumors or distant metastases. Differences in the platelet interactive phenotype between MDA-MB 435 parental cells and their tumor or metastasis derived variants were not due to changes in αvβ3 expression (Table 1). This suggests that integrin αvβ3 is expressed in these breast cancer cell variants in distinct activation states. These can be defined by the platelet interactive phenotype.

Our data are consistent with the idea that tumor cells which express platelet interactive αvβ3 are present

in the parental MDA-MB 435 cell line at a low frequency, and that these are selected *in vivo* during tumor growth and metastasis. It has been reported that the MDA-MB 435 cell line represents a polyclonal population, and that variants from distant metastases in mice are oligo- or monoclonal [10,11]. We therefore tested whether cells that express the platelet interactive phenotype can be selected *in vitro* from the MDA-MB 435 parental cell population, based on their ability to undergo platelet mediated arrest during blood flow. The cells were suspended in normal donor blood and perfused over a thrombogenic collagen I matrix under sterile conditions. Attached cells were expanded and resorted four times to enrich cells with a platelet interactive phenotype. Analytical perfusion experiments, in the absence or presence of function blocking anti αvβ3 antibody, showed that five independently sorted variant cell populations expressed the platelet interactive form of integrin αvβ3, and that the extent of platelet interaction is similar to that observed in the *in vivo* selected metastatic variants (Fig. 3A shows two examples). The expression levels of integrin αvβ3 were similar in the parental cell population and its *in vitro* selected variants (Fig. 3B). Analytical perfusion experiments revealed that all *in vitro* isolated platelet interactive variants stably expressed this phenotype over more than fifteen passages in culture. This confirms that the MDA-MB 435 parental cell line contains cells which express αvβ3 in either of two activation states, the platelet interactive or the non-interactive state. Unless under selective pressure, as during tumor growth or metastasis, the parental MDA-MB 435 cell population conserved the ratio of cells expressing the non-platelet interactive versus the interactive form of αvβ3. This was evident from repeated analytical blood perfusion experiments with parental MDA-MB 435 cells over more than 20 culture passages, during which the population at large maintained the non-platelet interactive phenotype.

We established a correlation between the platelet interactive and the metastatic phenotype of MDA-MB 435 breast cancer cells. We now sought to determine if there is a causal link between these two phenomena. To test the hypothesis that the activated, platelet interactive form of tumor cell integrin αvβ3, but not the non-activated form, promotes hematogenous metastasis, MDA-MB 435 cells were transfected with a β3 mutant to force expression of constitutively activated αvβ3. To accomplish this, a β3-minus variant was selected from MDA-MB 435 parental cells by exposing the cells to a saporin-anti β3 antibody conjugate to selectively kill cells that express β3 [12]. After five rounds of selection, a β3 minus population was obtained which maintained this phenotype over multiple culture passages (Fig. 4A). These cells were stably transfected with cDNA encoding either full length human β3 wild type, $\beta3_{WT}$, or mutant $\beta3_{D723R}$. The $\beta3_{D723R}$ mutant was shown to force platelet integrin αIIbβ3 into a constitutively active form [13] and to dimerize with the αv subunit which resulted in an altered functional state of integrin αvβ3 [14]. Here, stable transfectants expressed $\alpha v\beta3_{WT}$ or mutant $\alpha v\beta3_{D723R}$ at levels comparable to that of αvβ3 in the parental MDA-MB 435 cell line (Fig. 4A). These cells were analyzed for their ability to arrest in a platelet dependent manner during blood flow *in vitro*. Cells expressing mutant $\alpha v\beta3_{D723R}$, but not those expressing wild type αvβ3 or lacking β3, displayed the platelet interactive phenotype to the same extent as *in vivo* selected metastatic MDA-MB 435 cell variants (Fig. 4B).

The ability of integrin αvβ3 to support breast cancer cell arrest during blood flow in one functional form, but not the other, indicates strongly that αvβ3 exists in an activated and a non- or less-activated state in these tumor cells. To test whether the arrest competent form of breast cancer cell integrin αvβ3 supports other cell functions differently than the non-arrest competent form, we analyzed binding of the ligand-mimetic antibody WOW-1. WOW-1 is a genetically engineered Fab fragment that contains an RGD sequence in the context of the adenovirus penton base protein and serves as a monovalent ligand for αv integrins [15]. Importantly, WOW-1 was generated on the framework of the PAC-1 Fab which

recognizes platelet integrin αIIbβ3 in an activation dependent manner [16]. Therefore, WOW-1 specifically reports an activated state of integrin αvβ3 [15,17]. Here, we show that the MDA-MB 435 breast cancer cell variant that expresses arrest competent $\alpha v\beta 3_{D723R}$ bound twice as much WOW-1 than the variant expressing non-arrest competent $\alpha v\beta 3_{WT}$ (Fig. 5). Both cell variants expressed αvβ3 at equivalent levels (Fig 4). In the presence of Mn^{2+}, WOW-1 binding increased 2-fold in $\alpha v\beta 3_{D723R}$ expressing cells, but 5-fold in $\alpha v\beta 3_{WT}$ expressing cells. This indicates that $\alpha v\beta 3_{D723R}$ in these breast cancer cells is already in a state of increased activation in the absence of exogenous agonists.

It has been reported that integrin αvβ3 can exist in multiple functional states that promote cell migration differentially in a ligand specific manner [18]. To confirm the activated state of integrin αvβ3 in the arrest competent variants of the MDA-MB 435 breast cancer cell model, we therefore analyzed cell migration towards extracellular matrix proteins. We tested fibronectin, vitronectin and fibrinogen, which are all ligands for αvβ3 and can support cell adhesion in an αvβ3 dependent manner [19,20]. αvβ3 supported breast cancer cell migration towards fibronectin, because the αvβ3 expressing variants of the MDA-MB 435 cell model migrated 5-times more actively towards this ligand than the β3-lacking variant (Fig. 6). This was independent of the activation state of αvβ3, because cell variants expressing activated αvβ3 and those expressing less- or non-activated αvβ3 migrated equally well towards fibronectin. The migration measured in the β3-lacking cell variant was likely mediated by integrin α5β1, which is expressed in all tested cell variants (not shown). In contrast to fibronectin, breast cancer cell migration towards vitronectin was affected by the activation state of αvβ3. The cell variants expressing activated, arrest competent αvβ3 showed a significant increase in migratory activity towards vitronectin compared to the cell variants expressing the non-activated receptor (the *in vivo* selected metastatic variant 'Lung' and the $\alpha v\beta 3_{D723R}$ transfectant compared to parental cells and the $\alpha v\beta 3_{WT}$ transfectant) (Fig. 6). The low level of migration observed in the β3-lacking variant is likely supported by αvβ5, an integrin that all of the MDA-MB 435 cell variants expressed at equivalent levels (not shown). Fibrinogen did not support migration of any of the cell variants (Fig. 6).

To analyze whether activation of tumor cell integrin αvβ3 affects metastatic activity of breast cancer cells, MDA-MB 435 transfectants expressing either non-activated αvβ3 or constitutively activated mutant $\alpha v\beta 3_{D723R}$ were injected into SCID mice. The ability of the cells to colonize the lungs was compared to that of the β3 lacking cell variant. Metastatic activity was significantly enhanced ($p < 0.0001$) in cells expressing mutant $\alpha v\beta 3_{D723R}$ compared to cells expressing $\alpha v\beta 3_{WT}$ or no β3 (Fig. 7). There was no difference between the latter two groups. Thus, in the MDA-MB 435 breast cancer cell line, expression of activated αvβ3 results in a platelet interactive and strongly metastatic phenotype.

These results demonstrate, that human breast cancer cells can exhibit a platelet interactive and metastatic phenotype that is controlled by the activation state of tumor cell integrin αvβ3. This is consistent with a 'two hit hypothesis' [21,22] where αvβ3 expression is necessary, but not sufficient for successful breast cancer metastasis. Rather, additional as yet undefined factor(s) that control the activation state of the integrin are required for metastatic dissemination.

To test a significance of breast cancer cell integrin αvβ3 activation in human metastatic breast cancer, we isolated metastatic cells from specimens of patients with stage IV breast cancer. The tumor cells were isolated either from pleural effusions or peripheral blood samples. Table 2 gives an overview over our new cell lines established from these specimens. The cells were characterized as breast cancer cells

based on morphological and immunological criteria using a panel of marker antigens that are established for differential diagnosis in breast cancer pathology. All primary metastatic breast cancer cells were analyzed at early passages for integrin expression and the functional state of integrin αvβ3 based on the ability of the receptor to mediate tumor cell arrest during blood perfusion. Importantly, all primary metastatic breast cancer cells expressed αvβ3 in the activated functional state. The results for PE02JA cells, isolated from a pleural effusion are shown in Fig 1 and 2. The integrin expression profiles for BCM1, BCM2 and BMS cells are shown in Fig 8, 9 and 10. The adhesive activity of BCM1, BCM2 and BMS cells during blood perfusion was similar as shown for PE-2JA. With these primary human breast cancer cells, we demonstrate an association between the expression of integrin αvβ3 in a functionally activated state and the metastatic phenotype that has significance in clinical breast cancer.

Together, our results from the first year of funding respond to the originally proposed tasks in the statement or work:

Task 1. Analyze the metastatic potential of adhesive variants of the MDA-MB 435 human breast cancer cell line that express the activated or the resting form of integrin αvβ3
MDA-MB 435 breast cancer cell variants that express integrin αvβ3 in an activated functional state (MDA-MB 435 $\beta3_{D723R}$) are significantly more metastatic than variants that lack β3 expression (MDA-MB 435 $\beta3^-$) or express αvβ3 in a non-activated functional state (MBA-MB 435 $\beta3_{WT}$).

Task 2. Analyze the modulation of integrin αvβ3 function in adhesive variants of MDA-MB 435 breast cancer cells
We identified a mutation in the β3 integrin subunit as a mechanism that controls the functional state of MDA-MB 435 breast cancer cells. Transfection of our β3-lacking cell variant (MDA-MB 435$\beta3^-$) with a β3 gene carrying a mutation in the cytoplasmic hinge region, $\beta3_{D723R}$, resulted in expression of αvβ3 in a functionally activated state. In contrast to transfectants expressing $\alpha v\beta3_{WT,}$ cells expressing the mutant receptor $\alpha v\beta3_{D723R}$ were able to arrest during blood flow, had a significantly higher capacity to bind a ligand-mimetic antibody and showed an enhanced ability to migrate toward a vitronectin substrate.

Task 3. Test the significance of integrin αvβ3 activation in human breast cancer
Primary metastatic cells were isolated from patients with advanced breast cancer and were found to express integrin αvβ3 in a functionally activated state.

KEY RESEARCH ACCOMPLISHMENTS:

- We found that human breast cancer cells can express the adhesion receptor, integrin αvβ3 in an activated or a non-activated state.
- We documented that breast cancer cells, that express activated αvβ3, can arrest under blood flow conditions through interaction with platelets. This provides a potential mechanism for the attachment of circulating metastatic breast cancer cells within the circulation.
- We found a causal relationship between the expression of activated αvβ3 in breast cancer cells and a strongly increased metastatic phenotype in these tumor cells.

- We identified mutations in the β3 integrin subunit as a mechanism for integrin αvβ3 activation in human breast cancer cells.

- We showed that integrin αvβ3 activation has a significance in breast cancer patients, because we found the functionally activated state of αvβ3 expressed in primary metastatic breast cancer cells, isolated from peripheral blood or pleural effusions of patients with advanced breast cancer.

REPORTABLE OUTCOMES:

- *Manuscript:*
  B. Felding-Habermann, T.E. O'Toole, J.W. Smith, E. Fransvea, Z.M. Ruggeri, M.H. Ginsberg, P.E. Hughes, N. Pampori, S.J. Shattil, A. Saven, and B.M. Mueller (*in press)* Integrin activation controls metastasis in human breast cancer. Proc. Natl. Acad. Sci. USA

- *Abstracts:*
  Human breast cancer metastasis is affected by distinct functional forms of tumor cell integrin αvβ3. B. Felding-Habermann, TE O'Toole, ZM Ruggeri, JW Smith, MH Ginsberg, PE Hughes and BM Mueller. Keystone Symposium on Joint Regulation of Signaling Pathways by Integrins and Growth Factors. March 25-31, 2000, Breckenridge Colorado

  Integrin activation controls metastasis in human breast cancer. TE O'Toole, ZM Ruggeri, JW Smith, MH Ginsberg, PE Hughes and BM Mueller. Novartis/TSRI Joint Scientific Meeting, September 25-27, 2000. Monterey, California

- *Presentations:*
  Molecular aspects of tumor cell - platelet interaction during blood flow. Annual conference of the German and Austrian Societies of Hematology and Oncology. October 3-6, 1999, Jena, Germany
  .

  Integrin activation controls migration and invasion in breast cancer metastasis. Third Annual Retreat of the Department of Molecular and Experimental Medicine of TSRI, October 19, 2000, La Costa, California

- *Development of cell lines*

Cell Line	Isolated from	Characteristics
PE02JA	pleural effusion of a patient with stage IV breast cancer	metastatic breast cancer cells

PE10BC	pleural effusion of a patient with stage IV breast cancer	fibroblast
BCM1	peripheral blood of a patient with stage IV breast cancer	metastatic breast cancer cells
BCM2	peripheral blood of a patient with stage IV breast cancer	metastatic breast cancer cells
BMS	peripheral blood of a patient with stage IV breast cancer	metastatic breast cancer cells

- *Funding applied for based on work supported by this award*

  Modulation of Integrin Function in Tumor Metastasis. PI: Brunhilde Felding-Habermann, Funding agency: National Institutes of Health, Award type: RO1 Research Grant

CONCLUSIONS:

From the results generated in the past funding period, we conclude that human breast cancer cells can express the adhesion receptor integrin αvβ3 in an activated or a non-activated state. Most importantly, we found that integrin αvβ3 activation determines the metastatic phenotype of the tumor cells. Therefore, our results validate the original hypothesis. The results indicate a causal relationship between breast cancer cell integrin αvβ3activation and an increase in metastatic activity. They provide information on a potential mechanism of αvβ3 activation and show a significance of integrin αvβ3 activation in primary metastatic cells from breast cancer patients. Consequently, alterations within tumors that lead to the aberrant control of αvβ3 activation are expected to adversely affect the course of human breast cancer.

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APPENDICES:

Figure legends and Figures

Tables

Copy of the manuscript *in press*: Integrin activation controls metastasis in human breast cancer

Figure legends

Figure 1. Metastatic human breast cancer cells interact with platelets and arrest during blood flow. A. Variants of the MDA-MB 435 human breast carcinoma cell line (left panel) or freshly isolated metastatic cells from the pleural effusion of a patient with advanced breast cancer, PE02JA, (right panel) were analyzed for their ability to arrest at a collagen I matrix during blood perfusion. Metastatic PE02JA cells and MDA-MB 435 cell variants derived from mammary fat pad (mfp) tumors or metastases to the bone, lungs, lymphnode (LN) or pleural cavity in mice, but not the parental MDA-MB 435 cell population at large, bind to activated platelets and utilize this mechanism for cell arrest during blood flow. The tumor cells were stained with hydroethidine (red), suspended in normal donor blood containing mepacrine (green) and 50 nM H-D-Phe-Pro-Arg-chloromethyl ketone hydrochloride as anticoagulant. The suspension was perfused over a thrombogenic collagen I matrix at a venous wall shear rate of 50 sec^{-1} (4 dynes/cm^2) . Under these conditions, platelets attach to the matrix, become activated and form thrombi. During ongoing perfusion, tumor cell attachment to these thrombi was monitored by video microscopy and image acquisition at predefined positions with two distinct filter settings to discern platelet specific and tumor cell specific fluorescent signal. Thrombus formation and a negligible number of tumor cells that were directly attached to the matrix, independently of platelets, (not shown) were unaffected by the tumor cell type. Each column represents the mean of three analytical runs (± Stdev) using blood from the same donor. **B.** Projection of confocal sections through a heteroaggregate of platelets and the freshly isolated human metastatic breast cancer cells, PE02JA. The confocal images were acquired during ongoing perfusion of the tumor cell suspension in normal donor blood as detailed in A. While establishing contact with a thrombus, the tumor cells extended pseudopods for continued anchorage.

Figure 2. Platelet mediated breast cancer cell arrest depends on tumor cell integrin αvβ3. Mammary fat pad (mfp) tumor or metastasis derived variants of the MDA-MB 435 cell line or freshly isolated metastatic cells from the pleural effusion of a patient with advanced breast cancer, PE02JA, were analyzed as in Fig. 1 in the absence (open bars) or presence (hatched bars) of 80 μg/ml function blocking antibody. **A.** Anti-αvβ3 mab VNR1 27.1 inhibits tumor cell platelet interaction and thereby tumor cell arrest during blood flow. Thrombus formation was unaffected. **B.** Anti-αIIbβ3 mab LJ CP8 inhibits thrombus formation and abolishes tumor cell arrest during blood flow (mab effects on thrombus formation are not shown). Note that platelet integrin αIIbβ3 is not expressed on the breast cancer cells (not shown). Each column represents the mean of three analytical runs (± Stdev) using blood from the same donor.

Figure 3. MDA-MB 435 human breast cancer cells contain a subset that expresses activated αvβ3. The parental MDA-MB 435 breast cancer cell line contains tumor cells expressing activated integrin αvβ3, and these can be isolated *in vitro* based on their platelet interactive phenotype. MDA-MB 435 parental cells were suspended in normal donor blood and perfused as in Fig. 1, but under sterile conditions. Cells that underwent platelet mediated arrest were expanded and resorted four times. **A.** Two independently sorted polyclonal populations (05S05 and 10S05) were analyzed for their ability to undergo platelet mediated arrest during blood flow as in Fig. 1. in the absence (open bars) or presence

(hatched bars) of function blocking anti-integrin αvβ3 mab VNR1 27.1. Each column represents the mean of three analytical runs (± Stdev) using blood from the same donor. **B.** Parental MDA-MB 435 cells (Parent) and their *in vivo* (Lung met) or *in vitro* (05S05 and 10S05) selected variants express integrin αvβ3 at similar levels. Flow cytometric analysis of cells stained with mab LM609 (anti-αvβ3) (solid line) or isotype control mab (dotted line) followed by FITC-anti-mouse IgG antibodies (similar results were obtained with anti-αvβ3 mab VNR1 27.1).

Figure 4. Integrin αvβ3 activation results in the platelet interactive phenotype in MDA-MB 435 human breast carcinoma cells. A variant lacking β3 integrin expression (β3$^-$) was selected from the parental MDA-MB 435 cell line by repeated exposure to an anti-β3 saproin conjugate and stably transfected either with the β3 wild type gene ($\beta3_{WT}$) or the constitutively activated mutant $\beta3_{D723R}$. **A.** The transfectants expressed integrin αvβ3 at similar levels. Flow cytometric analysis of cells stained with anti-αv mab AV-8 (dashed line), anti-αvβ3 complex mab LM609 (solid line) or isotype control (dotted line) followed by FITC-anti-mouse IgG antibodies. **B.** Expression of constitutively activated $\alpha v\beta3_{D723R}$, but not $\alpha v\beta3_{WT}$, resulted in the platelet interactive phenotype in the MDA-MB 435 breast cancer cell model. MDA-MB 435 parental cells (Parent), their β3 lacking variant (β3$^-$), the transfectants ($\beta3_{WT}$, $\beta3_{D723R}$), or the *in vivo* selected metastatic variant (Lung) were suspended in normal donor blood, perfused and analyzed as in Fig. 1. Each column represents the mean of three analytical runs (± Stdev) using blood from the same donor.

Figure 5. Binding of the ligand-mimetic antibody Fab WOW-1 to functional variants of the MDA-MB 435 breast cancer cell model. Variants of the MDA-MB 435 breast cancer cell model that lacked β3 integrin expression (β_3^-) or were transfected either with the β3 wild type gene ($\beta3_{WT}$) or a constitutively activated β3 mutant gene ($\beta3_{D723R}$) were analyzed for binding of the activation dependent anti-αvβ3 antibody Fab WOW-1. The cells were incubated with 10 μg/ml WOW-1 in binding buffer contained 1mM $MgCl_2$ and 400 μM $CaCl_2$ and then with Alexa-anti mouse H+L conjugate. WOW-1 binding was measured by flow cytometry in the absence (open bars) or presence (hatched bars) of 250 μM $MnCl_2$ that was added to activate αvβ3. The data represent specific WOW-1 binding, defined as that inhibited by 2 mM RGDS peptide, and are presented as the means of duplicate analyses (± Stdev).

Figure 6. Haptotactic migration of functional variants of the MDA-MB 435 breast cancer cell model. Migration towards extracellular matrix proteins was analyzed for MDA-MB 435 parental cells (parent), an *in vivo* selected metastatic variant (Lung), or the β3-integrin lacking variant (β_3^-) and its transfectants expressing either the β3 wild type gene ($\beta3_{WT}$) or a constitutively activated β3 mutant gene ($\beta3_{D723R}$). Before the migration assay, the cells were starved over night in medium containing 0.5% fetal bovine serum and then seeded to the upper compartment of Transwell chambers. The undersides of the porous filters (8 μM pore size) were coated with human plasma fibronectin, vitronectin or fibrinogen and blocked. Bovine serum albumin was used as a negative control (not shown). Cells were allowed to migrated for 16 hrs at 37C and 5% CO_2. Migrated cells were counted at the under side of the filters after washing, removal of remaining cells from the upper side with damp cotton swaps, fixation and staining

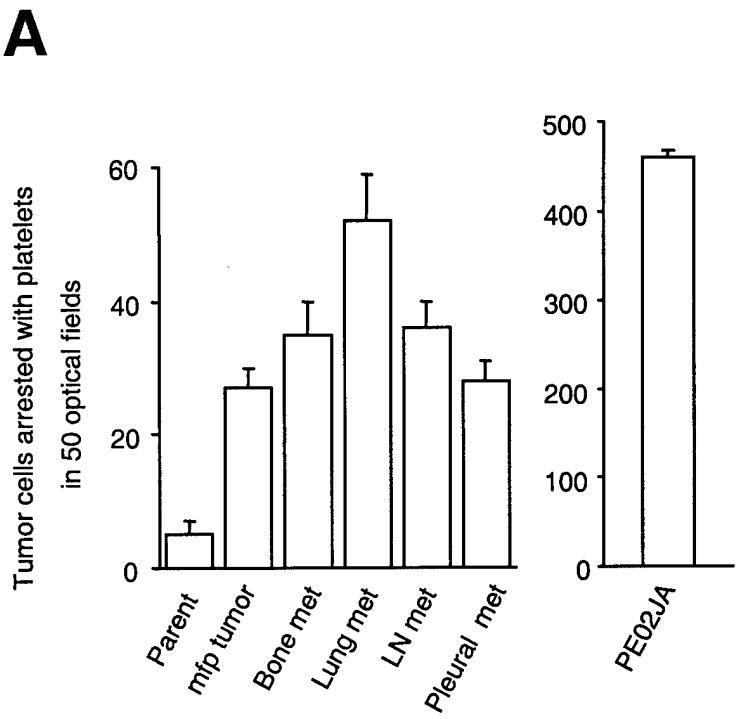
with DiffQuick. Each cell type and matrix protein was analyzed in duplicate. Migrated cells were counted in 10 random optical fields per filter in a blinded fashion by two observers. Each column represents the mean number of migrated cells per optical field of 20 counted fields (± Stdev). The migration data of metastatic cell variants compared to parental cells (upper panels) and the β3-lacking cells compared to its β3 transfectants (lower panels) are from independent experiments. Absolute numbers of migrated cells varied between experiments, but the ratios of migratory activities of the cell types remained constant in several independent experiments.

Figure 7. Integrin αvβ3 activation controls the metastatic potential in the MDA-MB 435 breast carcinoma cell model. A. Lungs of female C.B17/IcrTac *scid* mice 42 days after i.v. injection of $1x10^6$ tumor cells. Compared were the β3 integrin lacking cell variant and its transfectants expressing either $\alpha v\beta 3_{WT}$ or $\alpha v\beta 3_{D723R}$, as characterized in Fig. 4. The $\beta 3_{D723R}$ expressing variant had the platelet interactive phenotype and increased metastatic activity. **B.** Number of metastatic foci at the lung surface. Data points indicate the number of lung surface metastases for each animal and horizontal lines the median number of metastases per group (n=8). Cells expressing activated $\alpha v\beta 3_{D723R}$ produced a significantly larger number of metastases than cells lacking β3 or expressing non-activated $\alpha v\beta 3_{WT}$ ($p < 0.0001$ by the Kruskal Wallis test).

Figure 8. Integrin expression by primary metastatic human breast cancer cells, BCM1. BCM1 cells were isolated from a peripheral blood sample of a patient with stage IV breast cancer. The cells were isolated by immuno-magnetic bead sorting using a monoclonal antibody directed against a cell surface epithelial antigen (Ber-EP4). The cells were analyzed by flow cytometry for integrin expression as soon as enough cells were accumulated for the test. Anti-integrin antibodies were LM609 (αvβ3), LJ-CP8 (αIIbβ3), 15F11 (αvβ5), GoH3 (α6), AA3 (β4), 12F1 (α2), IIA1 (α5).

Figure 9. Integrin expression by primary metastatic human breast cancer cells, BCM2. BCM2 cells were isolated and analyzed by flow cytometry as in Fig 8.

Figure 10. Integrin expression by primary metastatic human breast cancer cells, BMS. BMS cells were isolated and analyzed by flow cytometry as in Fig 8.


A
Tumor cells arrested with platelets
in 50 optical fields
60
40
20
0
500
400
300
200
100
0
Parent
mfp tumor
Bone met
Lung met
LN met
Pleural met
PE02JA

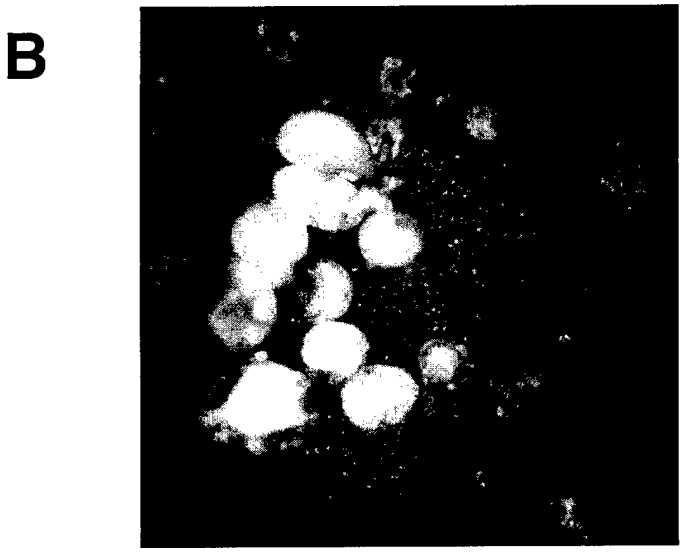
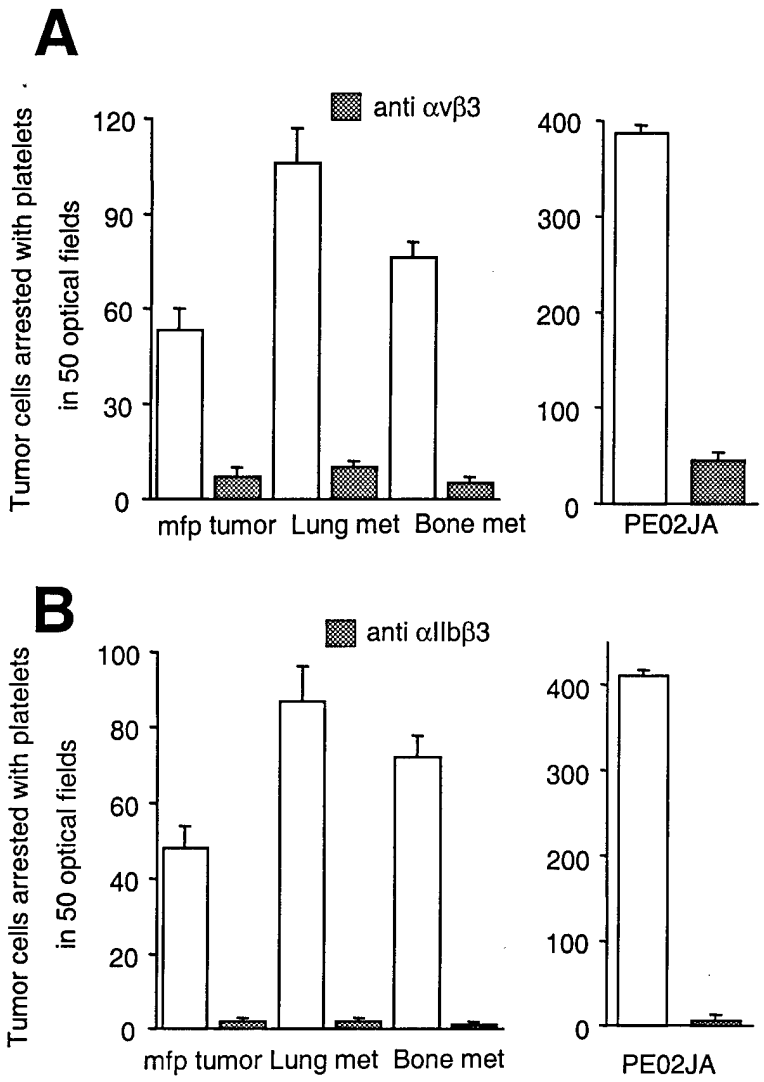

B

Figure 1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anti αvβ3
Tumor cells arrested with platelets in 50 optical fields
120
90
60
30
0
mfp tumor
Lung met
Bone met
400
300
200
100
0
PE02JA
B
anti αIIbβ3
Tumor cells arrested with platelets in 50 optical fields
100
80
60
40
20
0
mfp tumor
Lung met
Bone met
400
300
200
100
0
PE02JA

Figure 2

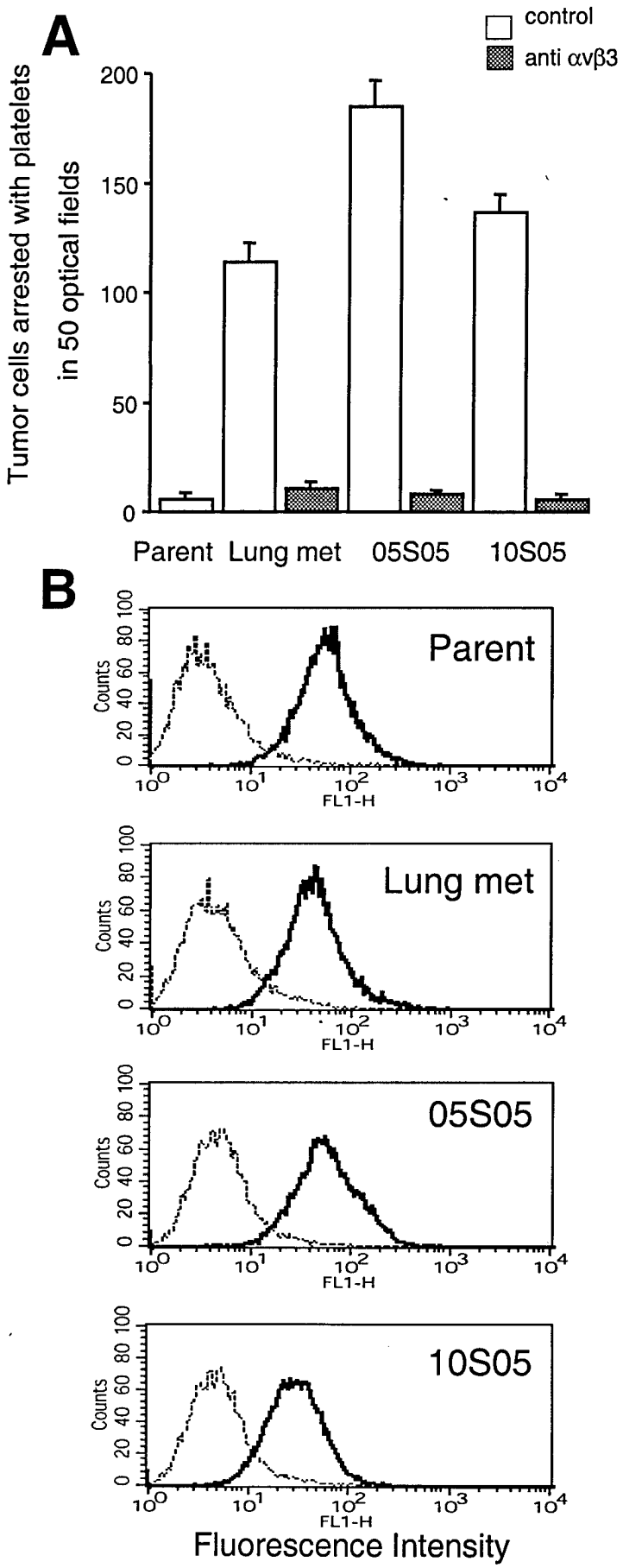
A
control
anti αvβ3
Tumor cells arrested with platelets in 50 optical fields
200
150
100
50
0
Parent
Lung met
05S05
10S05
B
Parent
Lung met
05S05
10S05
Counts
FL1-H
Fluorescence Intensity

Figure 3

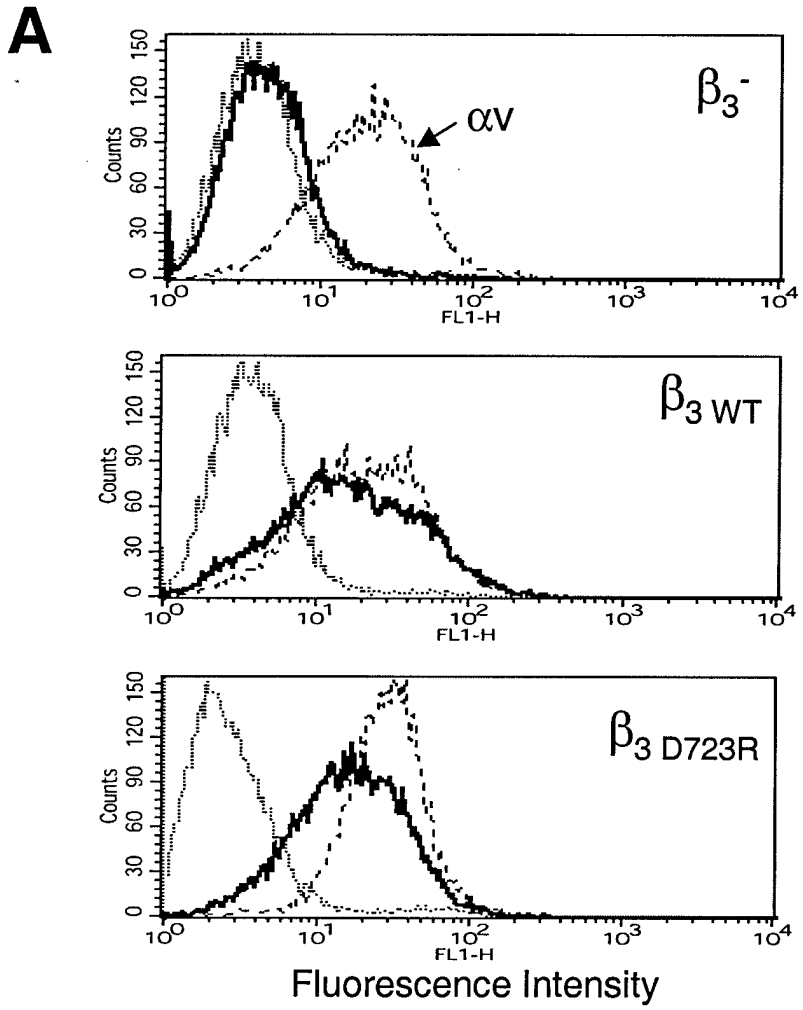
A
αv
β3-
β3 WT
β3 D723R
Counts
FL1-H
Fluorescence Intensity
B
Tumor cells arrested with platelets
in 50 optical fields
Parent
β3-
β3 WT
β3 D723R
Lung

Figure 4

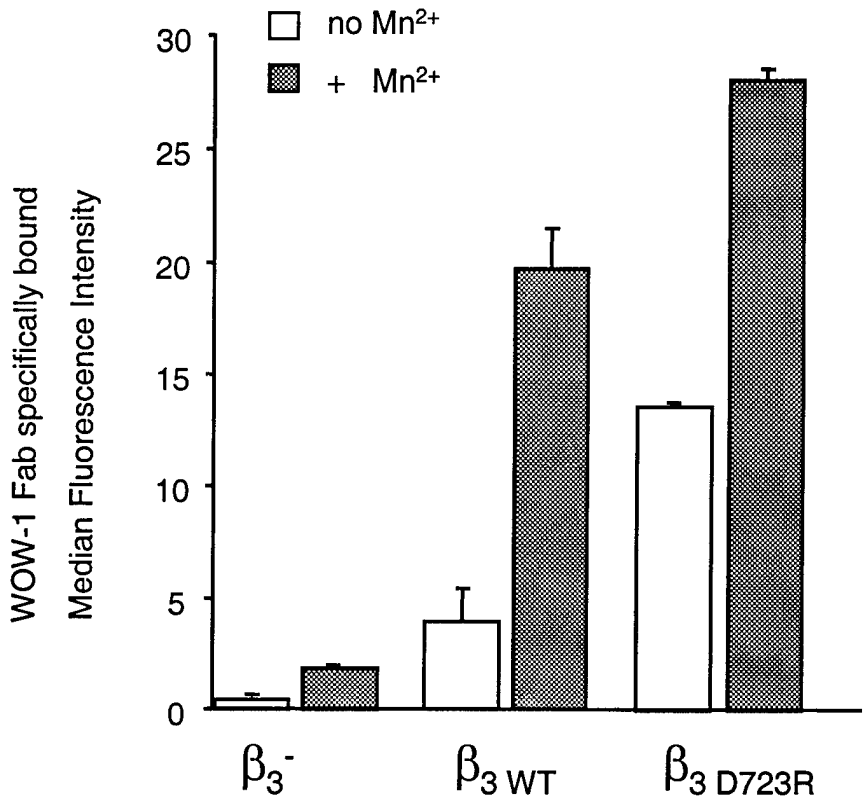
no Mn2+
+ Mn2+
WOW-1 Fab specifically bound
Median Fluorescence Intensity
30
25
20
15
10
5
0
β3-
β3 WT
β3 D723R

Figure 5

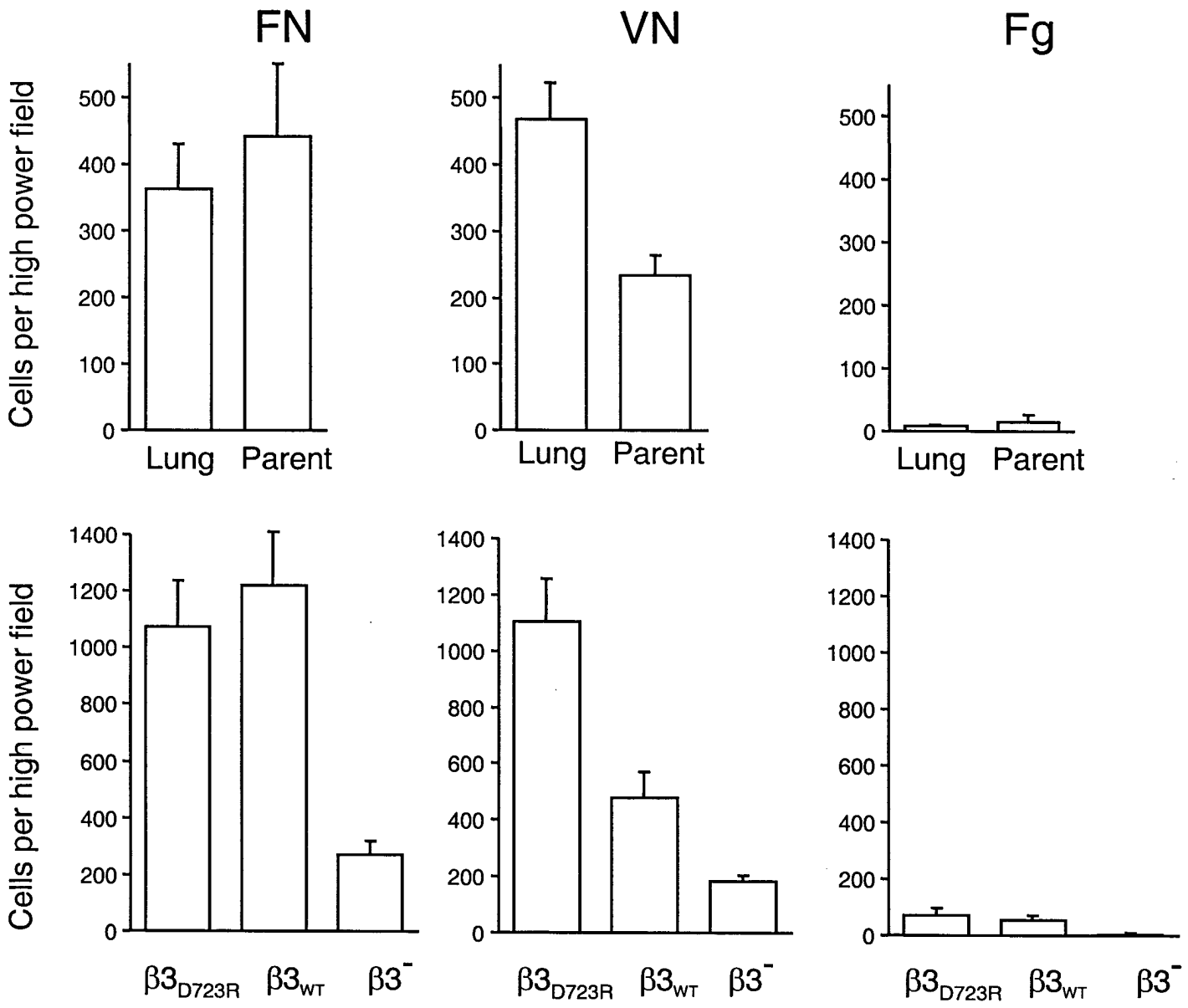
FN
VN
Fg
Cells per high power field
500
400
300
200
100
0
Lung
Parent
1400
1200
1000
800
600
400
200
0
β3D723R
β3WT
β3-

Figure 6

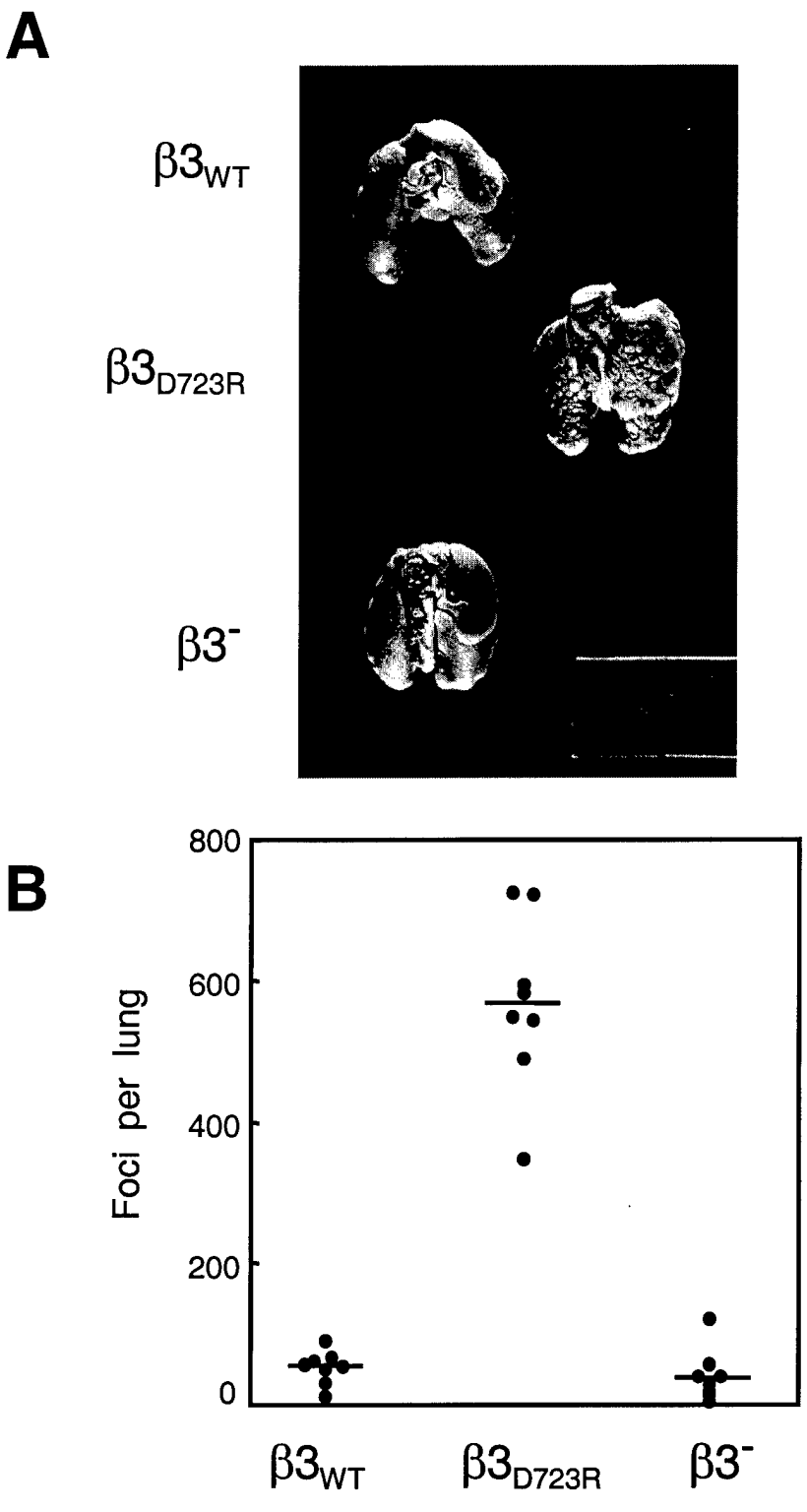
A
β3WT
β3D723R
β3-
B
Foci per lung
800
600
400
200
0
β3WT
β3D723R
β3-

Figure 7

Integrin Expression on Primary Metastatic Breast Cancer Cells

BCM-1

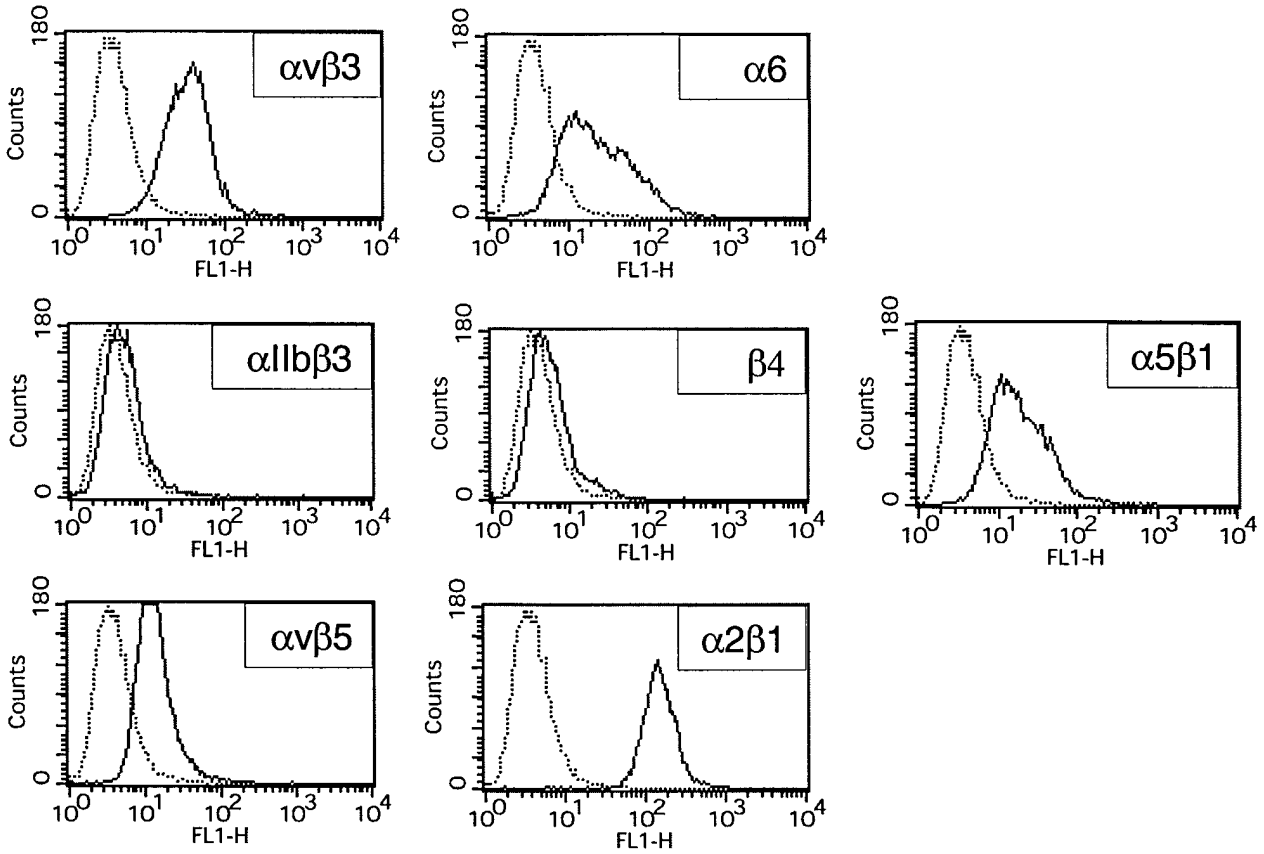

Figure 8

Integrin Expression on Primary Metastatic Breast Cancer Cells

BCM-2

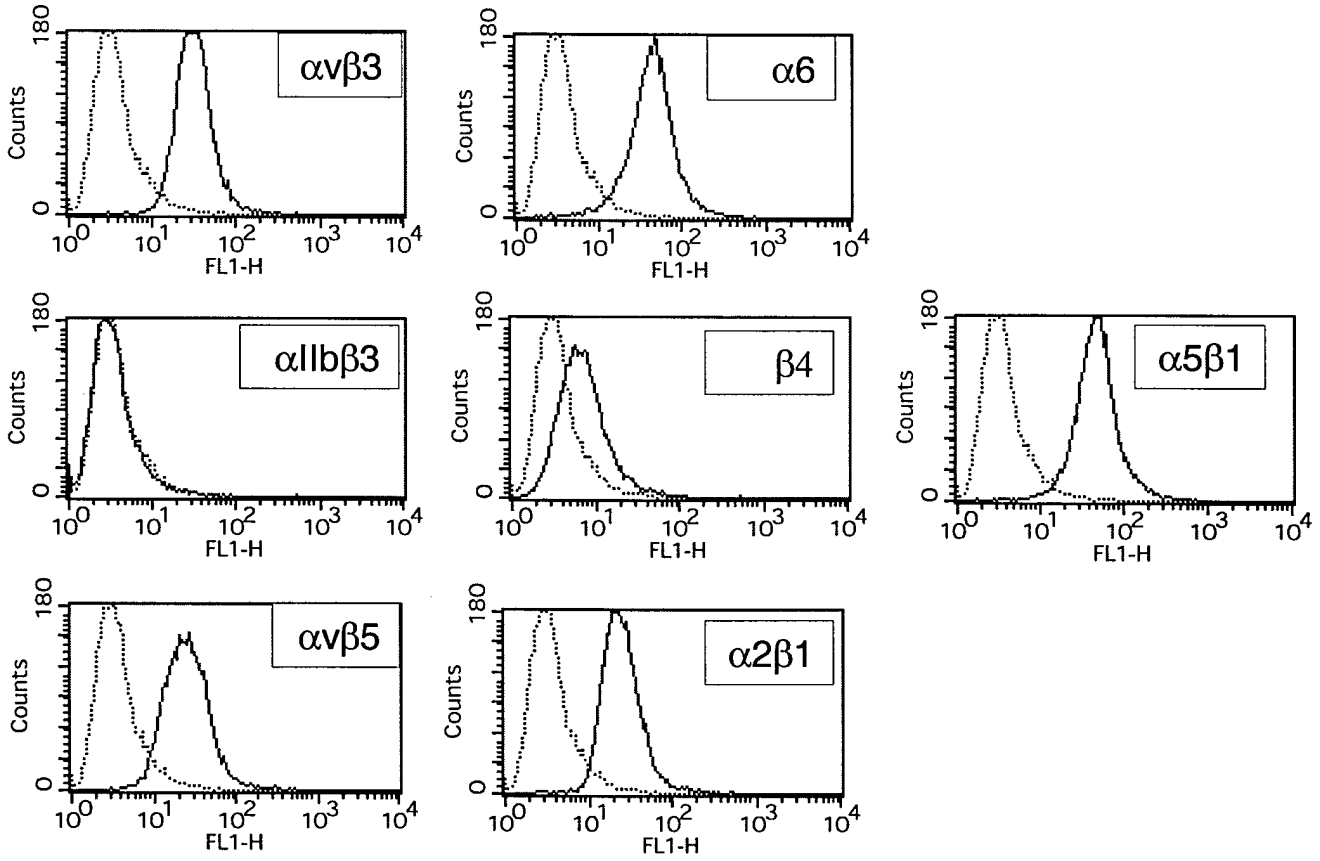

Figure 9

Integrin Expression on Primary Metastatic Breast Cancer Cells

BMS

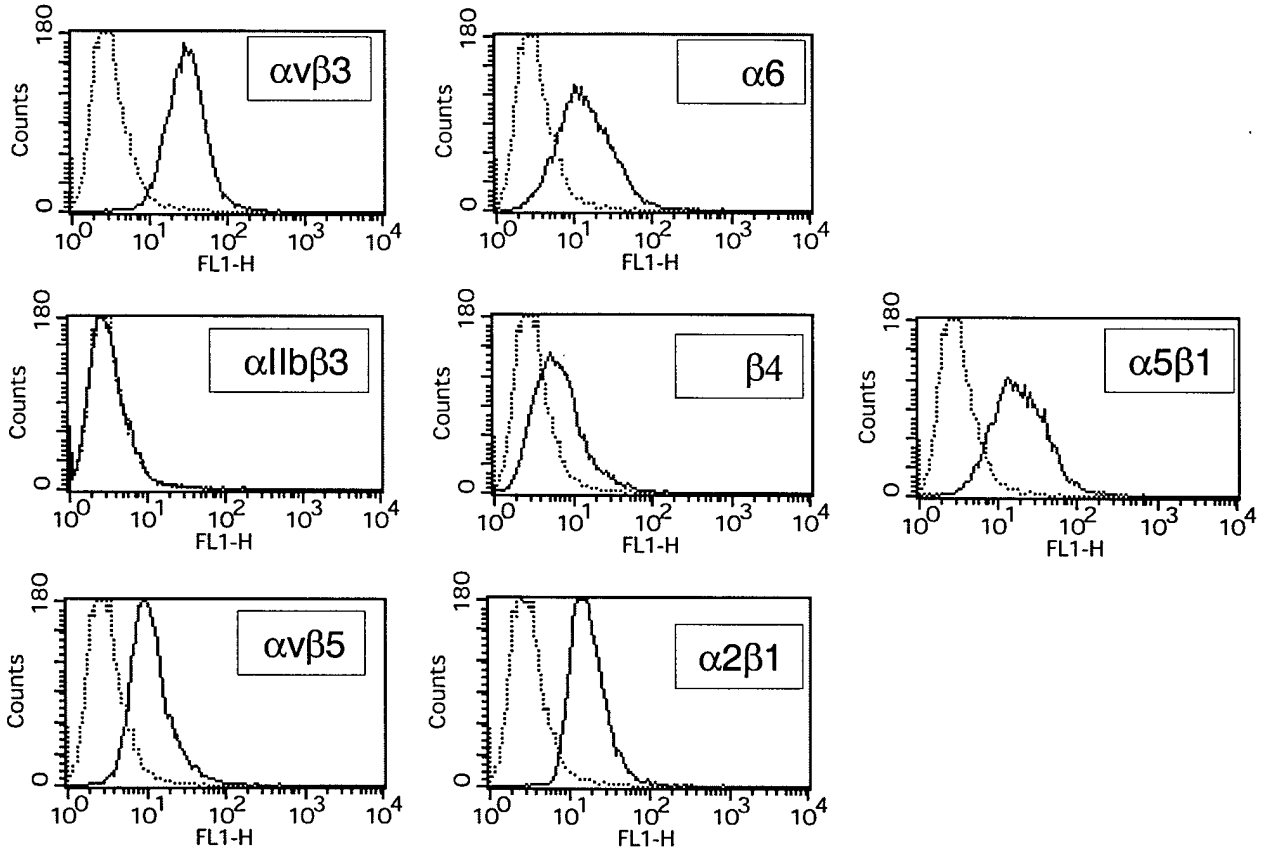

Figure 10

Table 1. Integrin αvβ3 expression in the human breast cancer cell model.
Variants of the MDA-MB 435 cell line were generated by injecting the parental cell line into the mammary fat pad (mfp) of SCID mice and culturing their descendants from developing tumors or distant metastases to bone, lungs, lymphnode or pleural cavity. PE02JA cells are human metastatic breast cancer cells, that were freshly isolated from the pleural effusion of a patient with advanced breast cancer. Integrin expression levels were determined by flow cytometry using the monoclonal antibodies LM609 (anti-αvβ3) or 12F1 (anti-α2) followed by FITC-anti-mouse IgG conjugate. Values are median fluorescence intensity.

	MDA-MB 435 Parent	MDA-MB 435 mfp Tumor	MDA-MB 435 Bone met	MDA-MB 435 Lung met	MDA-MB 435 Lymphnode met	MDA-MB 435 Pleural met	PE02JA
αvβ3	36.87	40.16	47.23	40.24	47.24	41.35	55.53
α2β1	28.42	26.37	22.63	28.54	20.34	28.70	235.37

Table 2. New cell lines established from specimens of patients with advanced breast cancer.
The cell lines were established from primary human tumor cells isolated from pleural effusions or peripheral blood samples of patients with stage IV breast cancer. Circulating breast cancer cells were isolated from blood samples by immuno-magnetic bead sorting. The beads were coated with a monoclonal antibody directed against a cell surface antigen, specific for epithelial cells (Ber-EP4). All cells were characterized based on morphological and immunological criteria. Marker antigens used for immunological characterization included a panel of

Cell Line	Isolated from	Characteristics	Integrin αvβ3 expression	Activation state of integrin αvβ3
PE02JA	pleural effusion of a patient with stage IV breast cancer	metastatic breast cancer cells	Yes	Activated
PE10BC	pleural effusion of a patient with stage IV breast cancer	fibroblast	Yes	N/D
BCM1	peripheral blood of a patient with stage IV breast cancer	metastatic breast cancer cells	Yes	Activated
BCM2	peripheral blood of a patient with stage IV breast cancer	metastatic breast cancer cells	Yes	Activated
BMS	peripheral blood of a patient with stage IV breast cancer	metastatic breast cancer cells	Yes	Activated

Major Classification: Biological Sciences *Minor Classification: Medical Sciences*

Integrin Activation Controls Metastasis in Human Breast Cancer

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Abstract

Metastasis is the primary cause of death in human breast cancer. Metastasis to bone, lungs, liver and brain involves dissemination of breast cancer cells via the blood stream and requires adhesion within the vasculature. In blood cells, adhesion within the vasculature depends on integrins, a family of transmembrane adhesion receptors, and is regulated by integrin activation. Here, we show that integrin αvβ3 can support breast cancer cell attachment under dynamic blood flow conditions in an activation dependent manner. Integrin αvβ3 was found to exist in two distinct functional states on human breast cancer cells. The activated, but not the non-activated state, supported tumor cell arrest during blood flow through interaction with platelets. Importantly, activated αvβ3 was found expressed by freshly isolated metastatic human breast cancer cells and variants of the MDA-MB 435 human breast cancer cell line, derived from mammary fat pad tumors or distant metastases in SCID mice. The expression of constitutively activated mutant $\alpha v\beta 3_{D723R}$, but not $\alpha v\beta 3_{WT}$, in a $\beta 3^-$ variant of the MDA-MB 435 cell system strongly promoted metastasis in the mouse model. Thus, breast cancer cells can exhibit a platelet interactive and metastatic phenotype that is controlled by the activation of integrin αvβ3. Consequently, alterations within tumors that lead to the aberrant control of integrin activation are expected to adversely affect the course of human breast cancer.

Introduction

Complications from metastatic disease are the primary cause of death in breast cancer (1). Metastasis to bone, lungs, liver and brain involves dissemination of tumor cells via the blood stream (2). This process depends on tumor cell intravasation, adhesion to the vessel wall, extravasation, infiltration and proliferation into target tissue. Many of these steps involve integrins, a family of transmembrane adhesion receptors composed of non covalently linked α and β subunits (3). Integrins are known to exist in distinct activation states, which exhibit different affinities for ligand. In general, integrin activation controls cell adhesion (4). Such control is particularly important in the vasculature where dynamic flow physically opposes cell attachment (5, 6).

Integrin αvβ3 has been implicated in the pathophysiology of malignant tumors (7). It clearly has a role on endothelial cells where it is required for tumor angiogenesis (8). In several malignancies, however, the αvβ3 complex was also found on the tumor cells, and expression correlates with tumor progression in melanoma, glioma, ovarian and breast cancer (9-13). In breast cancer, αvβ3 expression characterizes the metastatic phenotype as this integrin is clearly upregulated in invasive tumors, particularly in bone metastases (14). A mechanistic role of αvβ3 in the spread of breast cancer, however, has yet to be established. We previously suggested that an interaction of circulating tumor cells with platelets represents a potential mechanism for tumor cell arrest within the vasculature (15). Under dynamic blood flow conditions, shear forces physically oppose cell attachment. Therefore, cells must be equipped with specific adhesive mechanisms to support cell arrest (6). In leukocytes and platelets, attachment within the vasculature during inflammatory responses and thrombus formation is tightly regulated and

critically depends on the activation state of integrin adhesion receptors (16-19). It is currently unknown whether integrin activation controls tumor cell arrest in a similar manner. Here, we provide evidence that the ability of human breast cancer cells to attach during blood flow depends on the activation state of their integrin $\alpha v\beta 3$ and that this controls metastatic activity. Consequently, alterations within tumors that lead to the aberrant control of integrin activation are expected to adversely affect the course of human breast cancer.

Materials and Methods

Matrix proteins

Bovine fibrillar collagen I (Sigma, St. Louis) was used as a thrombogenic matrix in the blood perfusion studies. Suspensions of fibrillar collagen I were prepared in 0.5 M acetic acid, pH 2.8, homogenized at RT for 3 h, centrifuged at 1,000 rpm for 10 min to remove large particles and stored at 4°C. For matrix preparation, 200 µl of the collagen suspension was coated onto glass cover slips (No.1, 24 x 50 mm, Corning Inc., Corning, NY) and incubated in a humid atmosphere for 1h at RT. The cover slips were rinsed three times with DMEM before assembling the flow chamber (below). Vitronectin was purified from human plasma by affinity chromatography on heparin-Sepharose as described (20). Fibrinogen was purified from human plasma by glycine precipitation and gel filtration chromatography as described (15). Human plasma fibronectin was from (Collaborative Biochemical Products, Bedford, MA).

Antibodies

All antibodies were murine monoclonal IgGs except WOW-1, which is a recombinant Fab fragment (21). The IgGs were purified by affinity chromatography on Protein A-Sepharose. Mab VNR1 27.1 (anti-human αvβ3 complex) (22) was used to inhibit αvβ3 mediated breast cancer cell adhesive functions. Mab 15 (anti-human β3) (22) was conjugated to saporin to select β3-negative breast cancer cells (below). Mabs LM609 (anti-human αvβ3 complex) (23) (generously provided by Dr. D.A. Cheresh, The Scripps Research Institute, La Jolla), Tγ (anti-human thyroglobulin) (control IgG), AV-8 (anti-human αv) (15), 15F11 (anti-αvβ5) (24) and 12F1 (anti-human α2) (25) (generously provided by Dr. V.L. Woods, UCSD, La Jolla) were used to analyze

integrin expression by flow cytometry.

Cells and cell lines

The MDA-MB 435 human breast carcinoma cell line was a generous gift from Dr. J. E. Price (M.D. Anderson Cancer Center, Houston, Texas) (26). We derived variants from this cell line by injecting $5x10^5$ MDA-MB 435 parental cells into the mammary fat pads of adult female C.B 17/lcrTac *scid* mice (Taconic, Germantown, NY). After 8 weeks, mammary fat pad tumors were surgically removed, the mice allowed to recover and the tumors minced and cultured. Three weeks later, the mice were sacrificed, dissected sterilely, and metastases were recovered from bone, lungs, lymph nodes and the pleural cavity. The metastases were minced and cultured. PE02JA cells are metastatic human breast carcinoma cells that were freshly isolated from a discarded pleural effusion of a patient with advanced breast cancer. All cells were grown in EMEM supplemented with 10% fetal bovine serum, pyruvate, L-glutamine, vitamins and non-essential amino acids (BioWhittaker, Walkersville, MD).

Isolation of β3 integrin-negative MDA-MB 435 breast cancer cells

To isolate cells that lack β3-integrin expression from the parental MDA-MB 435 breast cancer cell line, β3 expressing cells were selectively killed by exposing them to an anti-β3 mab Ab15 - saporin conjugate (Ab15-Sap) (22, 27). MDA-MB 435 parental cells were exposed to increasing concentrations (0.512 pM and 40 nM) of Ab15-Sap in tissue culture medium containing 10% fetal calf serum. Three days later the cell viability was checked and the percentage of viable cells recorded. A concentration of 1.6 nM of Ab 15-Sap was found sufficient to kill the vast majority of the cells. To select cells that lack β3 integrin expression, the cells were exposed to 1.6 nM

Ab15-Sap for 3 days, allowed to expand in the absence of toxin conjugate for 4 days and then analyzed for β3-integrin expression by flow cytometry. Selective exposure to Ab15-Sap was repeated five times. After that, cells were expanded, analyzed for β3 expression, and aliquots were frozen at early passages. Lack of β3-integrin expression was routinely confirmed by flow cytometry during the course of this study.

Transfection

The β3-negative variant of the MDA-MB 435 breast cancer cell line was stably transfected with full length human β3 cDNA of either $\beta3_{WT}$ or mutant $\beta3_{D723R}$ (28). The β3 cDNAs were cloned into the pcDNA-1neo vector (Invitrogen, Carlsbad, CA) and introduced into the cells using the Lipofectamine transfection reagent (Gibco BRL, Rockville, Maryland). Stable transfectants were selected by exposure to 1.5 mg/ml G418 for 4 weeks. Integrin expression levels in β3- or mock-transfected cell populations were monitored by flow cytometry using antibodies directed to β3 or the αvβ3 complex. Cell populations expressing $\alpha v\beta3_{WT}$ or mutant $\alpha v\ \beta3_{D723R}$ at levels comparable to that of αvβ3 in the parental MDA-MB 435 breast cancer cell population were collected by sterile FACsorting.

Flow cytometry

MDA-MB 435 cell variants were harvested with PBS/EDTA. PE02JA cells, freshly isolated metastatic human breast cancer cells were harvested with trypsin. For flow cytometric analysis of integrin expression, the cells were incubated with 10 μg/ml monoclonal antibody in TBS containing 0.5% bovine serum albumin for 30 min on ice. The cells were washed in TBS and then stained with FITC goat-anti-mouse IgG conjugate (Zymed Laboratories, San Francisco, CA)

and analyzed using a Becton Dickinson FACScan. To measure binding of the ligand mimetic antibody Fab WOW-1 (21), variants of the MDA-MB 435 cell line were harvested with PBS/EDTA, washed once in binding buffer (137 mM NaCl, 2.7 mM KCl, 3.3 mM NaH_2PO_4, 3.8 mM HEPES, 1mM $MgCl_2$, 400 μM $CaCl_2$, 5.5 mM glucose, and 1 mg/ml bovine serum albumin, pH 7.4) and incubated with 10 μg/ml WOW-1 in the presence or absence of 250 μM $MnCl_2$ ± 2 mM RGDS peptide. The cells were incubated for 30 min at room temperature, washed with binding buffer, incubated with Alexa-anti-mouse IgG heavy and light chain conjugate (BIOSOURCE International, Camarillo, CA) for 30 min on ice, washed again and analyzed by flow cytometry.

Analytical perfusion studies

The ability of breast cancer cells to arrest during blood flow and to utilize an adhesive interaction with platelets in this process was measured as described previously (15). Briefly, tumor cells were suspended in normal donor blood and perfused over a collagen I matrix on glass cover slips at a venous wall shear rate of 50 sec^{-1} (4 dynes/cm^2). Adhesive events and cell interactions were visualized and recorded by fluorescence video or confocal laser microscopy (Zeiss LSM, Jena, Germany) and quantified by computer controlled image acquisition at 50 predefined positions during continued perfusion followed by image analysis (MetaMorph®, Universal Imaging Corp. West Chester, PA). MDA-MB 435 human breast cancer cells (29) or metastatic cells, freshly isolated from the pleural effusion of a patient with advanced breast cancer, were stained with hydroethidine (Polysciences, Inc. Warrington, PA) (red fluorescence) at 20 μg/ml final concentration for 30 min at 37 °C and washed twice to remove excess dye. The prestained tumor cells were suspended in normal donor blood anticoagulated with the thrombin inhibitor peptide

PPACK (H-D-Phe-Pro-Arg chloromethyl ketone hydrochloride, Bachem Bioscience Inc., Philadelphia, PA) at 50 nM and containing 10 μM mepacrine (quinancrine dihydrochloride) (Sigma, St. Louis, MO) (green fluorescence). Mepacrine concentrates in cell cytoplasmic and platelet dense granules. Thus, blood cells, tumor cells and platelets acquired green fluorescent staining and were visualized with filter settings at 488/515 nm (excitation/emission). The tumor cells were identified by their unique red fluorescence at 543/590 nm (excitation/emission). The contribution of tumor cell integrin αvβ3 and platelet integrin αIIbβ3 in the adhesive interaction was assessed in the absence or presence of 80 μg/ml of the function blocking antibodies mab VNR1 27.1 (22) or LJ CP8 (30), respectively. Antibody dose responses (20 - 150 μg/ml) were tested in perfusion assays in an attempt to inhibit platelet dependent arrest of MDA-MB 435 variant cells from a lung metastase (MDA-MB 435 Lung). Maximal inhibition was recorded between 70 - 80 μg/ml. The non-function blocking mabs AV-8 (anti-αv) and AV-10 (anti-β3) were used as controls and did not interfere with platelet- or tumor cell arrest. All tested mabs were IgG_1.

Preparative perfusion studies to isolate platelet interactive breast cancer cells

To isolate functional variants of the parental MDA-MB 435 breast cancer cell line based on their ability to undergo platelet mediated tumor cell arrest during blood flow, the parental cells were suspended in normal donor blood (anticoagulated as above) and sterilely perfused over a collagen I matrix at the same wall shear rate as above (50 sec^{-1}). At the end of the perfusion ($1x10^7$ tumor cells in 5 ml blood), unbound cells were removed by gentle washing with PBS, and thrombus formation was monitored by phase contrast microscopy. The coverslips were cultured in the same medium that was routinely used for breast cancer cell culture (EMEM containing L-glutamine,

pyruvate, non essential amino acids, vitamins and 10 % FBS). During the initial culture period, attached blood cells and platelets decayed and were removed gently by media replacement twice weekly. After 3 weeks, attached tumor cells were identified as proliferating colonies and expanded in tissue culture. All cells from a given slide were pooled and resorted four more times to select breast cancer cells with the platelet interactive phenotype. We generated five independently sorted MDA-MB 435 cell variants. Their abilities to undergo platelet mediated arrest during blood perfusion were quantified as above.

Haptotactic migration assay

Migration of the breast cancer cell variants towards purified extracellular matrix proteins was analyzed in transwells (8μm pore size, Costar, Corning, NY). The undersides of the porous filters were coated with 10 μg/ml human plasma fibronectin, vitronectin or 20 μg/ml fibrinogen in PBS. Bovine serum albumin was used as a negative control. The transwells were blocked with 5% non-fat dry milk in PBS containing 0.2% Tween 20 for 2 hrs at room temperature. Before the assay, the cells were incubated overnight in tissue culture medium containing 0.5% fetal bovine serum, harvested with PBS/EDTA, washed once in migration buffer (EMEM medium) and seeded at $6x10^4$ cells/well (upper transwell chamber). Cells were allowed to migrated for 14 hrs at 37°C and 5% CO_2. To quantify migrated cells, the filters were washed twice, and cells remaining at the upper side of the filters were removed with damp cotton swaps. The filters were fixed and stained with DiffQuick, excised and mounted onto glass slides. Migrated cells at the underside of the filters were counted in 10 random optical fields per filter in a blinded fashion by two observers.

In vivo metastasis assay

To compare the metastatic potential of MDA-MB 435 breast cancer cell variants, $1x10^6$ tumor cells were injected into the lateral tail vein of 6 week old female C.B17/IcrTac *scid* mice (Taconic, Germantown, NY) (n=8). The mice were sacrificed 42 days later and dissected. Each animal was analyzed by gross examination. Metastasis to the lungs was quantified after the lungs were excised and fixed in Bouin's fixative. Metastatic foci were counted at the lung surface under a dissecting microscope.

Results and Discussion

Metastatic human breast cancer cells interact with platelets and arrest during blood flow. To test the hypothesis that tumor cell binding to platelets during blood flow is a critical property of metastatic tumor cells, we generated tumor- or metastasis-derived variants of the MDA-MB 435 human breast cancer cell line. Parental MDA-MB 435 cells were injected into the mammary fat pads (mfp) of SCID mice and variant cells were retrieved from the resulting tumors or distant metastases to lymphnodes, lungs, bone and the pleural cavity. These variant cells were compared for their ability to attach to activated platelets and to undergo platelet mediated arrest during blood perfusion *in vitro*. MDA-MB 435 parental cells largely failed to adhere or interact with platelets under dynamic blood flow conditions. In contrast, cell variants derived from mfp tumors or distant metastases readily adhered and used platelet interaction for cell arrest (Fig. 1A). Importantly, metastatic cells freshly isolated from a pleural effusion of a patient with advanced breast cancer, exhibited a strong platelet interactive phenotype. The cells attached readily to platelets and were incorporated into thrombi that formed at a collagen type I matrix during blood perfusion. The typical morphology of breast cancer cell binding to activated platelets during blood perfusion is shown in Figure 1B using the clinical sample. Tumor cells that bound to attached, activated platelets extended pseudopods and established shear resistant contact with thrombi. Therefore, a platelet interactive phenotype, that promoted tumor cell arrest during blood flow, correlated with a tumorigenic and metastatic phenotype in the tested human breast cancer cell model.

Activated αvβ3 supports platelet dependent breast cancer cell arrest during blood flow. We reported previously that an interaction of human melanoma cells and platelets during blood flow

can be mediated by tumor cell integrin αvβ3 and platelet integrin αIIbβ3 in the presence of connecting plasma proteins, such as fibrinogen (15). To analyze whether platelet supported arrest of tumor or metastasis derived human breast cancer cells depends on a similar mechanism, blood perfusion studies were carried out in the presence of function blocking anti αvβ3 or anti αIIbβ3 antibodies. Arrest of mfp tumor or metastasis derived MDA-MB 435 breast cancer cells, as well as that of freshly isolated metastatic human breast cancer cells was strongly inhibited by the anti-αvβ3 antibody (Fig. 2A) and abolished by the anti-platelet-αIIbβ3 antibody (Fig. 2B). Similar results were obtained for all arrest competent, platelet interactive variants of the MDA-MB 435 cell model. Therefore, tumor cell integrin αvβ3 can mediate breast cancer cell arrest during blood flow through an interaction with platelets.

To test whether the observed differences in the platelet interactive phenotype between MDA-MB 435 parental cells and their tumor or metastasis derived variants were due to changes in the expression of integrin αvβ3, αvβ3 expression levels were analyzed by flow cytometry. The median fluorescence intensities for αvβ3 expression in the tumor or metastasis derived MDA-MB 435 cell variants were only slightly higher (9 - 28%) than that of the parental cell line (Table 1). Therefore, we conclude that the functional activity of αvβ3 in the parental cell line differs from that of αvβ3 in the *in vivo* selected cell variants because the receptor largely failed to support arrest of parental cells during blood flow, but mediated platelet dependent arrest of the tumor- and metastasis-derived variants. The similarity in αvβ3 expression in the parental breast cancer cell line and its tumorigenic and metastatic variants, therefore, suggests that integrin αvβ3 is present in distinct states of activation. The activation states can be defined by the platelet interactive phenotype.

The parental MDA-MB 435 human breast cancer cell line contains cells that stably express activated αvβ3. Our data are consistent with the idea that tumor cells expressing platelet interactive αvβ3 are present in the parental MDA-MB 435 cell line at a low frequency, and that these were selected *in vivo* during tumor growth and metastasis. It has been reported that the MDA-MB 435 cell line represents a polyclonal population, and that its variants derived from distant metastases in mice are oligo- or monoclonal (31, 32). We therefore tested whether cells that express the platelet interactive phenotype can be selected *in vitro* from the MDA-MB 435 parental cell population, based on their ability to undergo platelet mediated arrest during blood flow. The cells were suspended in normal donor blood and perfused over a thrombogenic collagen I matrix under sterile conditions. Attached cells were expanded and resorted four times to enrich cells with a platelet interactive phenotype. Analytical perfusion experiments, in the absence or presence of function blocking anti-αvβ3 antibody, showed that five independently sorted variant cell populations all expressed the platelet interactive form of integrin αvβ3. The extent of platelet interaction was similar to that observed in the *in vivo* selected metastatic variants. Two *in vitro* sorted cell populations, 05S05 and 10S05, compared to the parental cell line and the *in vivo* selected metastatic variant from the lung are shown in Figure 3A. The expression levels of integrin αvβ3 were similar in the parental cell population and the *in vitro* selected variants (Fig. 3B). All *in vitro* isolated platelet interactive variants stably expressed this phenotype over more than fifteen passages in culture. This confirms that the MDA-MB 435 parental cell line contains cells which express αvβ3 in either of two activation states, a platelet interactive or a non-interactive state. Unless under selective pressure, as during tumor growth or metastasis, the parental MDA-MB 435 cell population conserved the ratio of cells expressing the non-platelet interactive versus the interactive form of αvβ3. This was evident from repeated

analytical blood perfusion experiments with parental MDA-MB 435 cells for more than 20 culture passages, during which the population at large maintained the non-platelet interactive phenotype.

Integrin αvβ3 activation results in the platelet interactive, arrest competent phenotype in MDA-MB 435 human breast cancer cells. We established a correlation between the platelet interactive and the metastatic phenotype of MDA-MB 435 breast cancer cells. We now sought to determine if there is a causal link between these two phenomena. To test the hypothesis that the activated, platelet interactive form of tumor cell integrin αvβ3, but not the non-activated form, promotes hematogenous metastasis, MDA-MB 435 cells were transfected with a β3 mutant to express constitutively activated αvβ3. To accomplish this, a β3-minus variant was selected from MDA-MB 435 parental cells by exposing the cells to a saporin-anti-β3 antibody conjugate that selectively killed β3-expressing cells (27). After five rounds of selection, a β3 minus population was obtained which maintained this phenotype over multiple culture passages (Fig. 4A, top panel). These cells were transfected stably with cDNA encoding either full length human β3 wild type, $\beta3_{WT}$, or mutant $\beta3_{D723R}$. The $\beta3_{D723R}$ mutant has been shown to force platelet integrin αIIbβ3 into a constitutively activated form (28) and to dimerize with the αv subunit, which resulted in an altered functional state of integrin αvβ3 (33). Here, stable transfectants were generated that expressed either $\alpha v\beta3_{WT}$ or mutant $\alpha v\beta3_{D723R}$ at levels comparable to that of αvβ3 in the parental MDA-MB 435 cell line (Fig. 4A). These cells were analyzed for their ability to arrest in a platelet dependent manner during blood flow *in vitro*. Cells expressing mutant $\alpha v\beta3_{D723R}$, but not those expressing either wild type $\alpha v\beta3_{WT}$ or no β3, displayed the platelet interactive phenotype to an extent similar to that of the *in vivo* selected metastatic MDA-MB 435 cell variants. The cells

utilized the interaction with platelets to arrest during blood flow (Fig. 4B). As αvβ3 is the only β3 integrin in the MDA-MB 435 cell model, the expression of mutant $\beta3_{D723R}$ in these breast cancer cells resulted in a functionally activated αvβ3 integrin that supported cell arrest under dynamic flow conditions.

Integrin αvβ3 activation promotes binding of a ligand-mimetic antibody and enhances breast cancer cell migration towards vitronectin. The ability of integrin αvβ3 to support breast cancer cell arrest during blood flow in one functional form, but not the other, indicates strongly that αvβ3 exists in an activated and a non- or less-activated state in these tumor cells. To test whether the arrest competent form of breast cancer cell integrin αvβ3 supports other cell functions differently than the non-arrest competent form, we analyzed binding of the ligand-mimetic antibody WOW-1. WOW-1 is a genetically engineered Fab fragment that contains an RGD sequence in the context of the adenovirus penton base protein and serves as a monovalent ligand for αv integrins (21). Importantly, WOW-1 was generated on the framework of the PAC-1 Fab which recognizes platelet integrin αIIbβ3 in an activation dependent manner (34). Therefore, WOW-1 specifically reports an activated state of integrin αvβ3 (21). Here, we show that the MDA-MB 435 breast cancer cell variant that expresses arrest competent $\alpha v\beta3_{D723R}$ bound twice as much WOW-1 than the variant expressing non-arrest competent $\alpha v\beta3_{WT}$ (Fig. 5). Both cell variants expressed αvβ3 at equivalent levels (Fig 4). In the presence of Mn^{2+}, WOW-1 binding increased 2-fold in $\alpha v\beta3_{D723R}$ expressing cells, but 5-fold in $\alpha v\beta3_{WT}$ expressing cells. This indicates that $\alpha v\beta3_{D723R}$ exists in these breast cancer cells in a state of increased activation in the absence of exogenous agonists. Similar results were obtained in a comparison of the MDA-MB 435 parental cell population and the *in vivo* selected metastatic variant from the lung (not

shown).

It has been reported that integrin αvβ3 can exist in multiple functional states that promote cell migration differentially in a ligand specific manner (35). To confirm the activated state of integrin αvβ3 in the arrest competent variants of the MDA-MB 435 breast cancer cell model, we therefore analyzed cell migration towards extracellular matrix proteins. We tested fibronectin, vitronectin and fibrinogen, which are all ligands for αvβ3 and can support cell adhesion in an αvβ3 dependent manner (36, 37). αvβ3 supported breast cancer cell migration towards fibronectin, because the αvβ3 expressing variants of the MDA-MB 435 cell model migrated 5-times more actively towards this ligand than the β3-lacking variant (Fig. 6). This was independent of the activation state of αvβ3, because cell variants expressing activated αvβ3 and those expressing less- or non-activated αvβ3 migrated equally well towards fibronectin. The migration measured in the β3-lacking cell variant was likely mediated by integrin α5β1, which is expressed in all tested cell variants (not shown). In contrast to fibronectin, breast cancer cell migration towards vitronectin was affected by the activation state of αvβ3. The cell variants expressing activated, arrest competent αvβ3 showed a significant increase in migratory activity towards vitronectin compared to the cell variants expressing the non-activated receptor (the *in vivo* selected metastatic variant 'Lung' and the $\alpha v\beta 3_{D723R}$ transfectant compared to parental cells and the $\alpha v\beta 3_{WT}$ transfectant) (Fig. 6). The low level of migration observed in the β3-lacking variant is likely supported by αvβ5, an integrin that all of the MDA-MB 435 cell variants expressed at equivalent levels (not shown). Fibrinogen did not support migration of any of the cell variants (Fig. 6).

Together, the activated state of integrin αvβ3, as defined by the platelet interactive, arrest competent phenotype was here confirmed by increased binding of a ligand-mimetic antibody and increased support cell migration towards vitronectin.

Integrin αvβ3 activation controls the metastatic potential in the MDA-MB 435 breast cancer cell model. To analyze whether activation of tumor cell integrin αvβ3 affects the metastatic activity of breast cancer cells, MDA-MB 435 transfectants expressing either non-activated $\alpha v\beta 3_{WT}$ or constitutively activated mutant $\alpha v\beta 3_{D723R}$ were injected into the circulation of SCID mice. The ability of the cells to colonize the lungs was compared to that of the β3-lacking cell variant. Metastatic activity was significantly enhanced ($p < 0.0001$) in cells expressing mutant $\alpha v\beta 3_{D723R}$ compared to cells expressing $\alpha v\beta 3_{WT}$ or no β3 (Fig. 7). There was no difference between the latter two groups. Thus, in the MDA-MB 435 breast cancer cell model, expression of activated αvβ3 resulted in a platelet interactive phenotype and strongly increased metastatic activity.

It is presently unknown whether an interaction between breast cancer cells and platelets within the host circulation is a rate limiting ability that determines metastatic activity. The interaction of the tumor cells with platelets during blood flow *in vitro* allowed us to identify a functionally activated state of tumor cell integrin αvβ3, that may promote metastasis through a combination of altered adhesive, migratory and other cell functions. The platelet interactive variants of the MDA-MB 435 cell model that we identified by perfusing the cells in human blood also interacted with murine platelets and underwent platelet-mediated arrest when perfused in murine blood (not shown). Therefore, it is possible that binding of the human breast cancer cells to murine platelets

promoted the metastatic activity of the tumor cells in the mouse model.

Together, we show that αvβ3 can exist in breast cancer cells in distinct functional states. The activated but not the non-activated state supported tumor cell arrest during blood flow through interaction with platelets. We established a correlation between the expression of activated αvβ3 and the metastatic phenotype in the MDA-MB 435 human breast cancer cell model and in freshly isolated metastatic cells from a breast cancer patient. Importantly, we documented a causal relationship between the expression of activated αvβ3 and the metastatic potential in MDA-MB 435 breast cancer cells because expression of constitutively activated mutant $\alpha v\beta 3_{D723R}$, but not $\alpha v\beta 3_{WT}$, resulted in a significant increase in metastatic activity. These results demonstrate that human breast cancer cells can exhibit a platelet interactive and metastatic phenotype that is controlled by the activation state of tumor cell integrin αvβ3. This is consistent with a 'two hit hypothesis' (22, 38) where αvβ3 expression is necessary, but not sufficient for successful breast cancer metastasis. Rather, additional as yet undefined factor(s) that control(s) the activation state of the integrin are required for metastatic dissemination. Consequently, alterations within tumors that lead to the aberrant control of integrin activation are expected to adversely affect the course of human breast cancer.

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Abbreviations

Ab15-Sap, anti-human β3 antibody AB15 conjugated to saporin

FBS, fetal bovine serum

LN, lymph node

mab, monoclonal antibody

met, metastasis

mfp, mammary fat pad

PBS, phosphate buffered saline

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Figure legends

Figure 1. Metastatic human breast cancer cells interact with platelets and arrest during blood flow. **A**. Variants of the MDA-MB 435 human breast carcinoma cell line (left panel) or freshly isolated metastatic cells from the pleural effusion of a patient with advanced breast cancer, PE02JA, (right panel) were analyzed for their ability to arrest at a collagen I matrix during blood perfusion. Metastatic PE02JA cells and MDA-MB 435 cell variants derived from mammary fat pad (mfp) tumors or metastases to the bone, lungs, lymphnode (LN) or pleural cavity in mice, but not the parental MDA-MB 435 cell population at large, bind to activated platelets and utilize this mechanism for cell arrest during blood flow. The tumor cells were stained with hydroethidine (red), suspended in normal donor blood containing mepacrine (green) and 50 nM H-D-Phe-Pro-Arg-chloromethyl ketone hydrochloride as anticoagulant. The suspension was perfused over a thrombogenic collagen I matrix at a venous wall shear rate of 50 sec^{-1} (4 dynes/cm^2) (15) . Under these conditions, platelets attach to the matrix, become activated and form thrombi. During ongoing perfusion, tumor cell attachment to these thrombi was monitored by video microscopy and image acquisition at predefined positions with two distinct filter settings to discern platelet specific and tumor cell specific fluorescent signal. Thrombus formation and a negligible number of tumor cells that were directly attached to the matrix, independently of platelets, (not shown) were unaffected by the tumor cell type. Each column represents the mean of three analytical runs (± Stdev) using blood from the same donor. **B.** Projection of confocal sections through a heteroaggregate of platelets and the freshly isolated human metastatic breast cancer cells, PE02JA. The confocal images were acquired during ongoing perfusion of the tumor cell suspension in normal donor blood as detailed in A. While establishing contact with a thrombus, the tumor cells extended pseudopods for continued

anchorage.

Figure 2. Platelet mediated breast cancer cell arrest depends on tumor cell integrin αvβ3. Mammary fat pad (mfp) tumor or metastasis derived variants of the MDA-MB 435 cell line or freshly isolated metastatic cells from the pleural effusion of a patient with advanced breast cancer, PE02JA, were analyzed as in Fig. 1 in the absence (open bars) or presence (hatched bars) of 80 μg/ml function blocking antibody. **A.** Anti-αvβ3 mab VNR1 27.1 (22) inhibits tumor cell platelet interaction and thereby tumor cell arrest during blood flow. Thrombus formation was unaffected. **B.** Anti-αIIbβ3 mab LJ CP8 inhibits thrombus formation and abolishes tumor cell arrest during blood flow (mab effects on thrombus formation are not shown). Note that platelet integrin αIIbβ3 is not expressed on the breast cancer cells (not shown). Each column represents the mean of three analytical runs (± Stdev) using blood from the same donor.

Figure 3. MDA-MB 435 human breast cancer cells contain a subset that expresses activated αvβ3. The parental MDA-MB 435 breast cancer cell line contains tumor cells expressing activated integrin αvβ3, and these can be isolated *in vitro* based on their platelet interactive phenotype. MDA-MB 435 parental cells were suspended in normal donor blood and perfused as in Fig. 1, but under sterile conditions. Cells that underwent platelet mediated arrest were expanded and resorted four times. **A.** Two independently sorted polyclonal populations (05S05 and 10S05) were analyzed for their ability to undergo platelet mediated arrest during blood flow as in Fig. 1. in the absence (open bars) or presence (hatched bars) of function blocking anti-integrin αvβ3 mab VNR1 27.1. Each column represents the mean of three analytical runs (± Stdev) using blood from the same donor. **B.** Parental MDA-MB 435 cells (Parent) and their *in*

vivo (Lung met) or *in vitro* (05S05 and 10S05) selected variants express integrin αvβ3 at similar levels. Flow cytometric analysis of cells stained with mab LM609 (anti-αvβ3) (solid line) or isotype control mab (dotted line) followed by FITC-anti-mouse IgG antibodies (similar results were obtained with anti-αvβ3 mab VNR1 27.1).

Figure 4. Integrin αvβ3 activation results in the platelet interactive phenotype in MDA-MB 435 human breast carcinoma cells. A variant lacking β3 integrin expression ($\beta3^-$) was selected from the parental MDA-MB 435 cell line by repeated exposure to an anti-β3 saproin conjugate and stably transfected either with the β3 wild type gene ($\beta3_{WT}$) or the constitutively activated mutant $\beta3_{D723R}$. **A.** The transfectants expressed integrin αvβ3 at similar levels. Flow cytometric analysis of cells stained with anti-αv mab AV-8 (dashed line) (15), anti-αvβ3 complex mab LM609 (solid line) or isotype control (dotted line) followed by FITC-anti-mouse IgG antibodies. **B.** Expression of constitutively activated $\alpha v\beta3_{D723R}$, but not $\alpha v\beta3_{WT}$, resulted in the platelet interactive phenotype in the MDA-MB 435 breast cancer cell model. MDA-MB 435 parental cells (Parent), their β3 lacking variant ($\beta3^-$), the transfectants ($\beta3_{WT}$, $\beta3_{D723R}$), or the *in vivo* selected metastatic variant (Lung) were suspended in normal donor blood, perfused and analyzed as in Fig. 1. Each column represents the mean of three analytical runs (± Stdev) using blood from the same donor.

Figure 5. Binding of the ligand-mimetic antibody Fab WOW-1 to functional variants of the MDA-MB 435 breast cancer cell model. Variants of the MDA-MB 435 breast cancer cell model that lacked β3 integrin expression (β_3^-) or were transfected either with the β3 wild type gene ($\beta3_{WT}$) or a constitutively activated β3 mutant gene ($\beta3_{D723R}$) were analyzed for binding of

the activation dependent anti-αvβ3 antibody Fab WOW-1. The cells were incubated with 10 μg/ml WOW-1 in binding buffer contained 1mM $MgCl_2$ and 400 μM $CaCl_2$ and then with Alexa-anti mouse H+L conjugate. WOW-1 binding was measured by flow cytometry in the absence (open bars) or presence (hatched bars) of 250 μM $MnCl_2$ that was added to activate αvβ3. The data represent specific WOW-1 binding, defined as that inhibited by 2 mM RGDS peptide, and are presented as the means of duplicate analyses (± Stdev).

Figure 6. Haptotactic migration of functional variants of the MDA-MB 435 breast cancer cell model. Migration towards extracellular matrix proteins was analyzed for MDA-MB 435 parental cells (parent), an *in vivo* selected metastatic variant (Lung), or the β3-integrin lacking variant (β_3^-) and its transfectants expressing either the β3 wild type gene ($\beta3_{WT}$) or a constitutively activated β3 mutant gene ($\beta3_{D723R}$). Before the migration assay, the cells were starved over night in medium containing 0.5% fetal bovine serum and then seeded ($6x10^4$/well) to the upper compartment of Transwell chambers. The undersides of the porous filters were coated with human plasma fibronectin (FN), vitronectin (VN) or fibrinogen (Fg) and blocked. Bovine serum albumin was used as a negative control (not shown). Cells were allowed to migrated for 14 hrs at 37°C and 5% CO_2. Migrated cells were counted at the under side of the filters after washing, removal of remaining cells from the upper side with damp cotton swaps, fixation and staining with DiffQuick. Each condition was analyzed in duplicate. Migrated cells were counted in 10 random optical fields per filter in a blinded fashion by two observers. Each column represents the mean number of migrated cells per optical field of 20 counted fields (± Stdev). The migration data of metastatic cell variants compared to parental cells (upper panels) and the β3-lacking cells compared to its β3 transfectants (lower panels) are from independent

experiments. Absolute numbers of migrated cells varied between experiments, but the ratios of migratory activities of the cell types remained constant in several independent experiments.

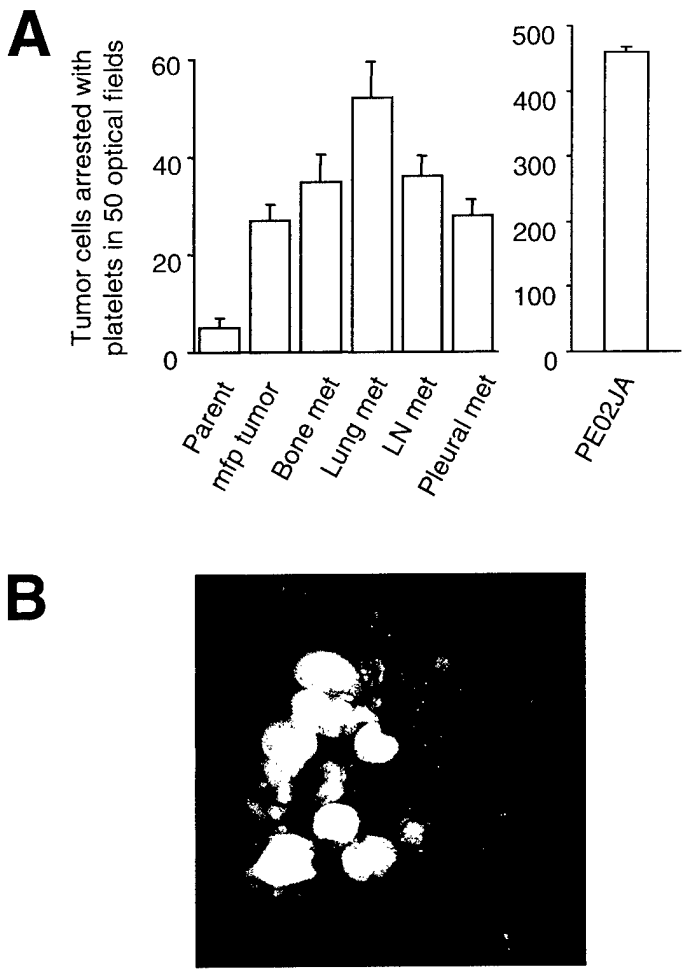
Figure 7. Integrin αvβ3 activation controls the metastatic potential in the MDA-MB 435 breast carcinoma cell model. A. Lungs of female C.B17/lcrTac *scid* mice 42 days after i.v. injection of $1x10^6$ tumor cells. Compared were the β3 integrin lacking cell variant and its transfectants expressing either $\alpha v\beta 3_{WT}$ or $\alpha v\beta 3_{D723R}$, as characterized in Fig. 4. The $\beta 3_{D723R}$ expressing variant had the platelet interactive phenotype and increased metastatic activity. **B.** Number of metastatic foci at the lung surface. Data points indicate the number of lung surface metastases for each animal and horizontal lines the median number of metastases per group (n=8). Cells expressing activated $\alpha v\beta 3_{D723R}$ produced a significantly larger number of metastases than cells lacking β3 or expressing non-activated $\alpha v\beta 3_{WT}$ ($p < 0.0001$ by the Kruskal Wallis test).

Table 1. Integrin αvβ3 expression in the human breast cancer cell model.

Variants of the MDA-MB 435 cell line were generated by injecting the parental cell line into the mammary fat pad (mfp) of SCID mice and culturing their descendants from developing tumors or distant metastases to bone, lungs, lymphnode or pleural cavity. PE02JA cells are human metastatic breast cancer cells that were freshly isolated from the pleural effusion of a patient with advanced breast cancer. Integrin expression levels were determined by flow cytometry using the monoclonal antibodies LM609 (anti-αvβ3) or 12F1 (anti-α2) followed by FITC-anti-mouse IgG conjugate. Values are median fluorescence intensity.

	MDA-MB 435 Parent	MDA-MB 435 mfp Tumor	MDA-MB 435 Bone met	MDA-MB 435 Lung met	MDA-MB 435 Lymphnode met	MDA-MB 435 Pleural met	PE02JA
αvβ3	36.87	40.16	47.23	40.24	47.24	41.35	55.53
α2β1	28.42	26.37	22.63	28.54	20.34	28.70	235.37

Figure 1


A
Tumor cells arrested with platelets in 50 optical fields
60
40
20
0
500
400
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200
100
0
Parent
mfp tumor
Bone met
Lung met
LN met
Pleural met
PE02JA
B

Figure 2

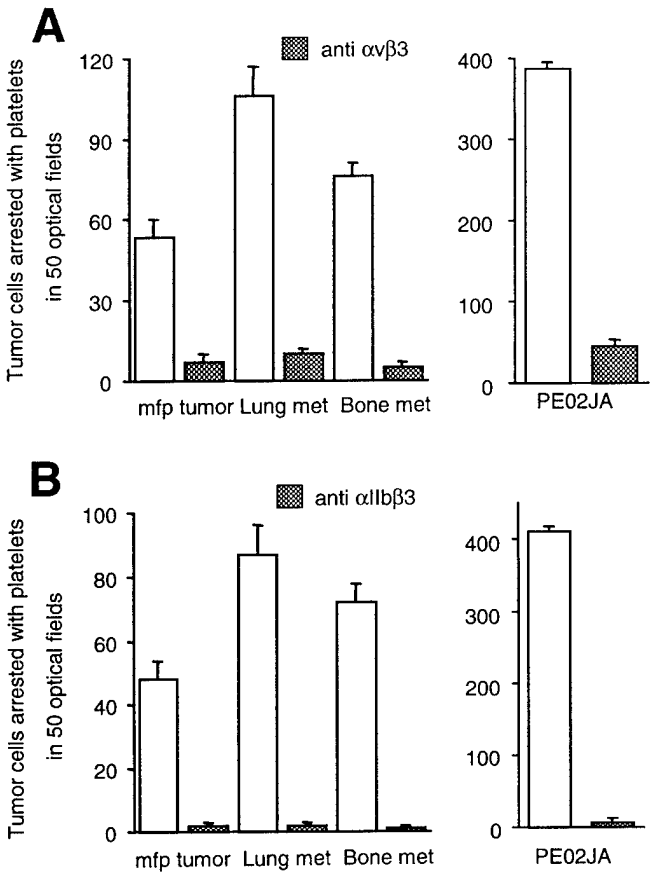

A
anti αvβ3
Tumor cells arrested with platelets in 50 optical fields
120
90
60
30
0
400
300
200
100
0
mfp tumor
Lung met
Bone met
PE02JA
B
anti αIIbβ3
Tumor cells arrested with platelets in 50 optical fields
100
80
60
40
20
0
400
300
200
100
0
mfp tumor
Lung met
Bone met
PE02JA

Figure 3

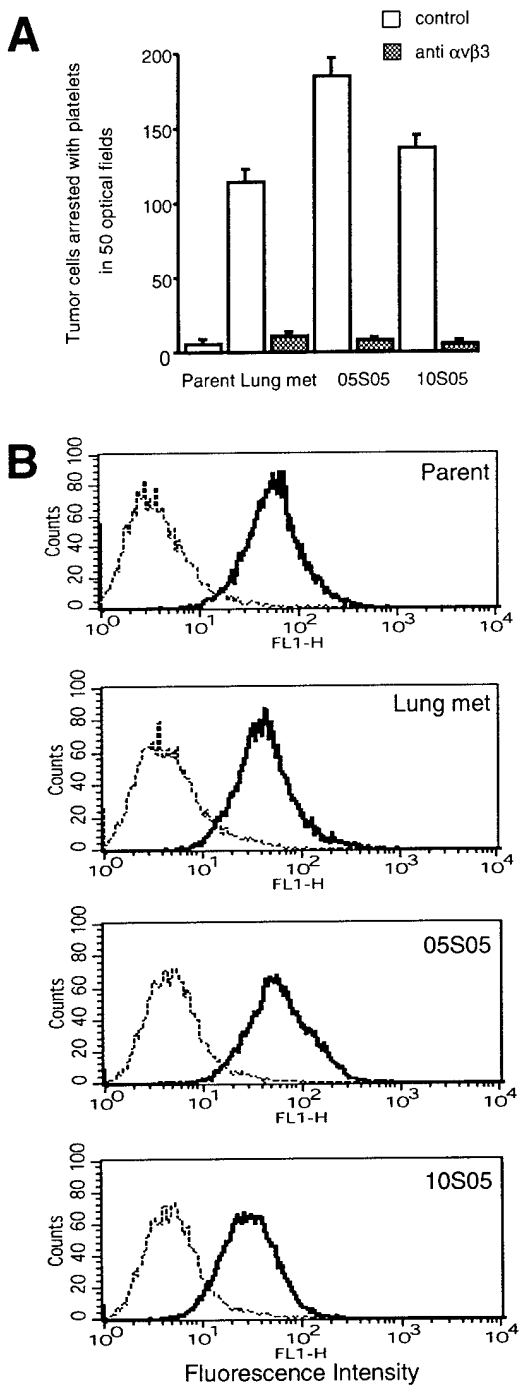

A
control
anti αvβ3
Tumor cells arrested with platelets in 50 optical fields
200
150
100
50
0
Parent
Lung met
05S05
10S05
B
Parent
Lung met
05S05
10S05
Counts
FL1-H
Fluorescence Intensity

Figure 4

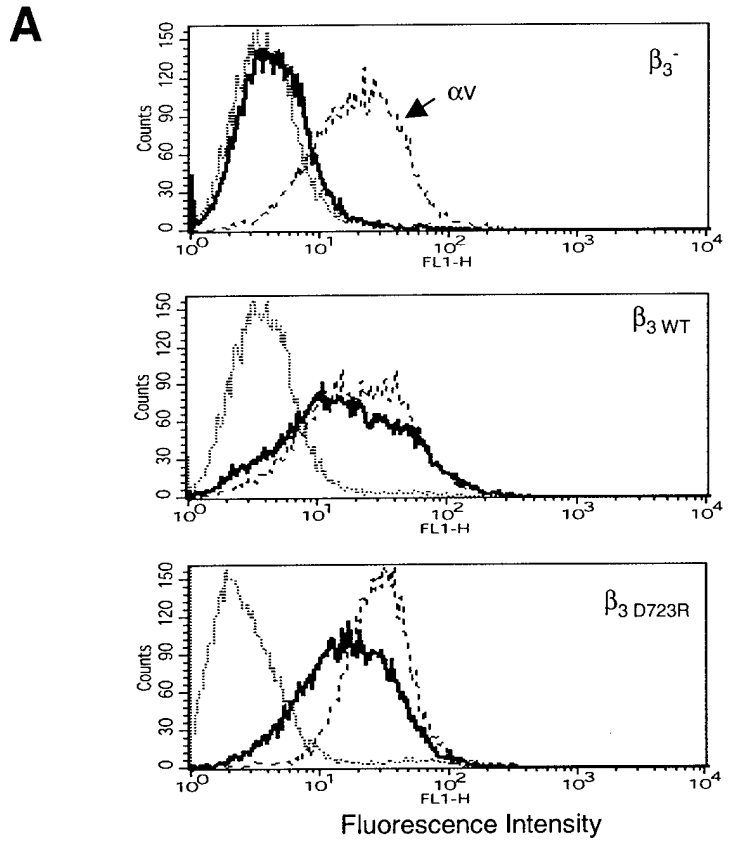

A
β3-
αV
β3 WT
β3 D723R
Counts
FL1-H
Fluorescence Intensity
B
Tumor cells arrested with platelets in 50 optical fields
Parent
β 3 -
β 3 WT
β 3 D723R
Lung

Figure 5

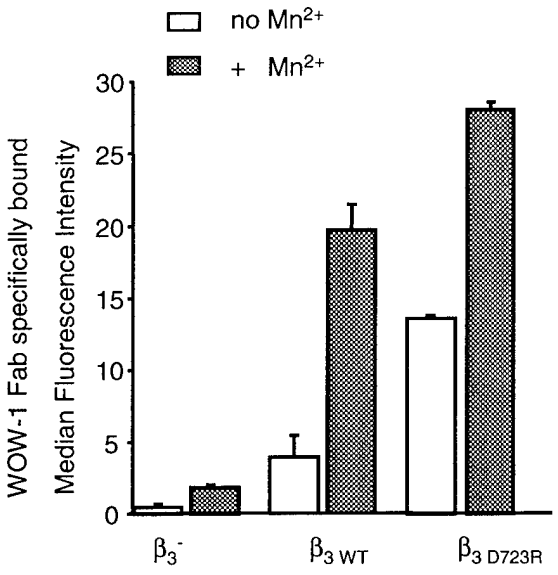

no Mn2+
+ Mn2+
WOW-1 Fab specifically bound
Median Fluorescence Intensity
30
25
20
15
10
5
0
β3-
β3 WT
β3 D723R

Figure 6

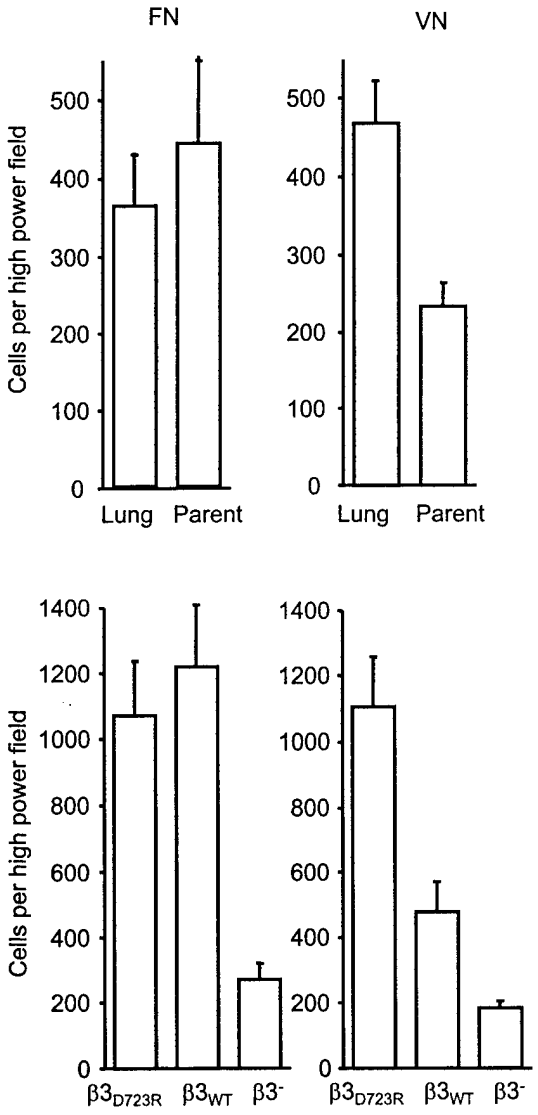
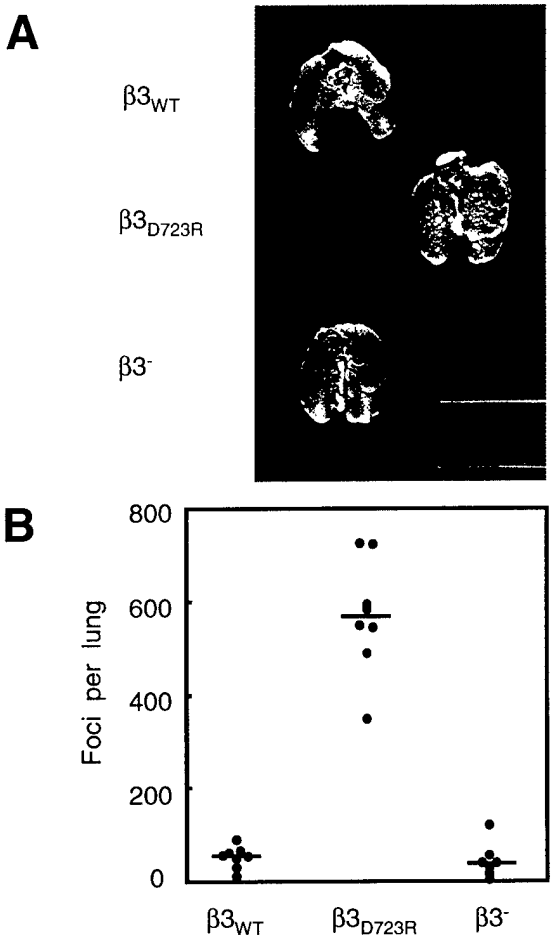

FN
VN
Cells per high power field
0
100
200
300
400
500
Lung
Parent
0
200
400
600
800
1000
1200
1400
β3D723R
β3WT
β3-

Figure 7


A
β3WT
β3D723R
β3-
B
800
600
400
200
0
Foci per lung
β3WT
β3D723R
β3-